Regulation of calcium-permeable AMPA receptors in glia

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Abstract

AMPA receptors (AMPARs) mediate majority of excitatory synaptic transmission, and are involved in fast neuronal glial signaling in the central nervous system (CNS). These receptors are tetrameric assemblies that function as homomeric or heteromeric combinations of four AMPAR subunits (GluA1-4). Many key AMPAR properties are determined by the GluA2 subunit, the absence of which renders the AMPAR calcium permeable. Calcium permeable AMPARs (CP-AMPARs) are relatively widespread in the CNS being present in certain neurons and many glia. The calcium permeability of neuronal AMPARs is known to be relatively plastic, changing during development and following high frequency synaptic activity. It is clear that calcium permeable AMPARs play an important role in normal functions of Bergmann glia and oligodendrocyte precursor cells, however factors that regulate the CP-AMPAR in glial cells are still poorly understood.

Little is known about the transmembrane auxiliary AMPA subunit (TARPs) present in glial cells. In **Chapter 3** of this thesis, I consider the role of transmembrane AMPAR regulatory proteins (TARPs) in controlling AMPAR channel properties and trafficking. In particular, these experiments identify γ-5 as a novel TARP that occurs mainly in glial cells, and is present at high levels in Bergmann glia. Functionally, this TARP appears selective for long form AMPAR subunits (predominantly calcium permeable). My experiments also demonstrate that γ-5 co-localizes with a late endosome protein. Uniquely, γ-5 appears selective for the trafficking of GluA2, and is dependent on the protein SAP97 for trafficking and/or localisation.

Oligodendrocyte precursor cells (OPCs), which are responsible for the formation of myelinating cells in the CNS, form a large proportion of the glial cells present in developing brain. OPCs are vulnerable to cell death in conditions such as periventricular white matter damage (hypoxic-ischemic white matter injury) in newborns. **In Chapter 4**, I have investigated factors that regulate calcium permeable AMPARs in OPCs, including mGluRs and purinergic receptors. Surprisingly I find that these receptors regulate calcium permeable AMPARs. This chapter identifies key mechanisms underlying this switch in AMPAR subtype, including the TARP γ-2 (Stargazin) that is involved in mGluR-induced 'plasticity' of AMPARs in OPCs. The experiments described in the final chapter, investigates the transmembrane AMPAR regulatory protein cornichon-3 (CNIH3). In this thesis I identified that the AMPAR regulatory protein cornichon-3 (CNIH3) is expressed at the cell surface in OPCs and that when over-expressed can influence their AMPAR channel properties.

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Introduction

The majority of neuronal and glial communication in the central nervous system (CNS) is mediated by the excitatory neurotransmitter glutamate, which activates the ionotropic AMPA, kainate and NMDA receptors and the metabotropic glutamate receptors (mGluRs). The modification of glutamate receptors underlies several plasticity changes in the CNS, including long-term potentiation (LTP), long-term depression (LTD) that have been linked to memory formation.

LTP was first described in the hippocampus. The simple structure of the hippocampus enabled *in vivo* extracellular records of synaptic events for long periods (Bliss and Gardner-Medwin, 1973). Hippocampal LTP increases the strength of synaptic efficacy, which is associated with increased trafficking of AMPA receptors (AMPARs) to the synapse. This increased trafficking of AMPARs has been extensively characterised using electrophysiological, biochemical and molecular techniques (Luscher et al., 2000).

Persistent low frequency synaptic stimulation can result in LTD, which has been well-characterised at the parallel fibre to Purkinje cell synapses (PF-PC) in the cerebellum and in the CA1 hippocampus. In CA1 hippocampal cells, LTD can be induced by long periods of low frequency synaptic stimulation (1-3 Hz, 5-15 min) of the CA3 Schaffer collateral axons. PF-PC LTD can also mimicked by applying the mGluR1 and mGluR5 agonist (R,S)-dihydroxyphenylglycine (DHPG) to external bathing solution for approximately 5–10 minutes (Bolshakov and Siegelbaum, 1994; Huber et al., 2000; Manahan-Vaughan, 2000).

Activation of mGluR1 on stellate cell interneurons of the cerebellum and neurons of the ventral tegmental area (VTA) results in a persistent increase in the insertion of synaptic calcium-impermeable AMPARs into the plasma membrane (Kelly et al., 2009; Mameli et al., 2007).

Bergmann glia and oligodendrocyte precursor cells (OPCs) express calcium permeable AMPA receptors, which are important for regulation of synaptic communication with neurons. (Bellamy and Ogden, 2005) previously identified AMPA mediated plasticity in Bergmann glial cells (BGCs) after the stimulation of parallel fibers (PF) in the cerebellum. Recent studies have identified synaptic connections between neurons and oligodendrocyte precursor cells (OPCs), which have been shown to undergo changes in cell surface expression of CP-AMPARs after neuronal activation (Bergles et al., 2000; Ge et al., 2006). However the mechanisms that regulate this change in cell surface CP-AMPA expression in OPCs are not well understood.

In this introduction I will be briefly summarizing some of the relevant basic molecular biology of AMPARs and their regulation by the transmembrane AMPA regulatory proteins (TARPs). I will also be describing briefly the key features of the G-protein coupled receptors mGluRs and P2Ys and their role in neuronal-glial communication. Finally I will describe background information about Bergmann glia and oligodendrocyte precursor cells.

1.1. Glutamate receptors

To date, three main classes of ionotropic glutamate receptors (iGluRs) have been identified: N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate receptors (AMPARs) and kainate receptors (KARs). Glutamate is also a ligand for the metabotropic

glutamate receptors (mGluRs), which are important G-protein coupled receptors that are classified into 8 different subtypes (mGluR1-8).

NMDARs were identified in the 1970s and eventually cloned in 1991, when studies revealed three families of receptors: GluN1, GluN2 and GluN3 (formally NR1-NR3) (Moriyoshi et al., 1991). Between 1989 and 1992, AMPARs were first cloned and were found to have 4 distinct receptor subunits: GluA1, GluA2, GluA3 and GluA4 (formally GluR1-4) (Hollmann and Heinemann, 1994) (Collingridge et al., 2009). KARs were identified in the 1990s and were found to have 5 subunits: GluK1, GluK2, GluK3, GluK4 and GluK5 (formally GluR5-7 and KA1-KA2) (Bettler et al., 1990). GluK1, GluK2, GluK3, and GluK4 all have high affinity for glutamate. However, while GluK1, GluK2 and GluK3 can form functional ion channels in the CNS, GluK4 and GluK5 cannot (Werner et al., 1991).

1.1.1. AMPARs

The structure of AMPARs has been elucidated through the use of x-ray crystallography and biochemical studies. The four identified AMPAR subunits (GluA1-4) share a 70% nucleotide sequence homology and are each approximately 100 kDa in molecular weight (Rogers et al., 1991).

1.1.2. AMPAR structure

AMPARs contain three transmembrane domains (M1, M3 and M4; M2 is a cytoplasmic re-entrant loop). Research suggests that the N-terminal domain of AMPARs is important for the regulation of AMPAR assembly (Kuusinen et al., 1999). In studies where the N-terminus of an AMPAR was exchanged with the N-terminus of kainate receptors, multiple properties of AMPARs were changed, including glutamate binding affinity and desensitisation kinetics (Stern-Bach et al., 1994; Stern-Bach et al., 1998).

High-resolution examination of X-ray crystal structures of AMPAR treated with the agonist kainate reveals that the extracellular domain of AMPARs contains the ligand-binding domain (LBD) (**Figure 1.1B**). Glutamate binding regions in the LBD were found to be in a conserved amino acid binding pocket (S1 and S2). The binding pocket is linked to the transmembrane domains (M1 and M3-4) in a clam shell-like binding core (Armstrong and Gouaux, 2000). Exchanging the ligand-binding domains of GluA3 with the ligand-binding domains of GluK2 resulted in profound changes to the affinity of glutamate for GluA3, highlighting the importance of the LBD in controlling the binding properties of agonists (Stern-Bach et al., 1994).

1.1.3. Alternative splicing, editing and post-translational modification

Splice variants of AMPARs for GluA2 and GluA4 occur at the C-terminus. Each variant results in the expression of either “long” or “short” form receptors. Short-form GluA2 and long-form GluA4 are the predominantly expressed forms in the CNS. Short-form GluA4 (GluA4c) have been observed in cerebellar granule cells (Gallo et al., 1992; Kohler et al., 1994). There are no identified splice variants for the short-form GluA3 or the long-form GluA1 (**Figure 1.1**). All AMPAR genes encode two different splice variants in the extracellular loop between M3 and M4, known as ‘flip’ and ‘flop’ (Monyer et al., 1991; Pei et al., 2009). The main functional difference between the flip and flop AMPARs are their desensitisation properties in response to prolonged glutamate activation; flop receptors desensitise more rapidly than flip receptors (Sommer et al., 1990). It is possible to delay the onset of desensitisation of flip AMPARs by applying the drugs cyclothiazide or aniracetam. Desensitisation of flop AMPAR isoforms can

be delayed with the addition of phenyoxyacetamide derivative PEPA (Kessler et al., 2000; Partin et al., 1994).

The mRNA of GluA2 AMPAR subunit undergoes editing in the pore-lining region. Pore domain-editing results in the exchange of an uncharged glutamine with a positively charged arginine (Q/R) and renders GluA2R containing AMPA channels impermeable to Ca^{2+} (Burnashev et al., 1992b; Geiger et al., 1995). This editing is achieved through the enzymatic action of the RNA-dependent adenosine deaminase 2, resulting in 99% editing of GluA2 receptors in the adult brain.

Increase in single-channel conductance is observed when an edited GluA2R is removed from AMPARs (Swanson et al., 1997). There are also significant changes to the whole-cell AMPARs rectification index (RI) measured from expression systems transfected with GluA2 containing AMPARs, producing a characteristic linear I/V relationship. The removal of GluA2R from AMPARs and an incorporation of CP-AMPARs produce an inwardly rectifying response (**Figure 1.2**).

Defective editing of GluA2 receptors can result in the expression of GluA2Q and thereby lead to the excessive entry of Ca^{2+} into neurons, which has been implicated has been in a number of diseases including amyotrophic lateral sclerosis (ALS), stroke, Huntington's disease, Alzheimer's disease, malignant gliomas (Hideyama et al.; Rui et al., 2010; van Vuurden et al., 2009).

AMPARs are further edited at the R/G site, which is controlled by RNA splicing of the flip/flop region, and results in alteration of the desensitisation properties of AMPARs. The unedited R form receptors desensitise more slowly than the edited G form (Seeburg et al., 1998).

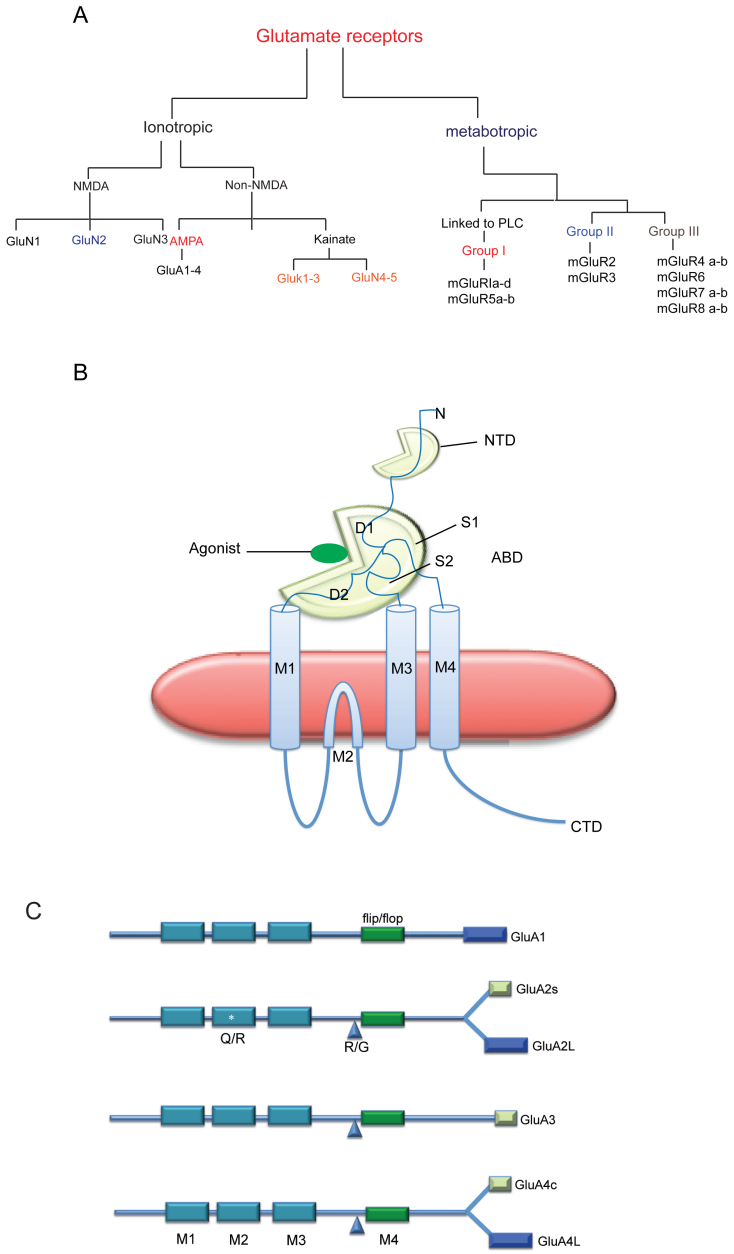


Figure 1.1. Glutamate receptor classification and topology.

(A) Glutamate receptor classification and their receptor subtypes. **(B)** AMPARs contain 3 transmembrane domains (M1, M3 and M4) with a ligand-binding domain (S1 and S2) and an extracellular N-terminal (NTD) and intracellular C-terminal domain (CTD). **(C)** AMPARs have flip and flop variants between the M3 and M4 region that modify the properties of the receptors. Alternative splice variants of differing lengths in the C-terminus from GluA2 and GluA4. RNA editing may also occur within the M2 region at the Q/R site of GluA2, resulting in the formation of a calcium-impermeable GluA2R. Illustrations adapted from (Hansen et al., 2007) and (Dingledine et al., 1999).

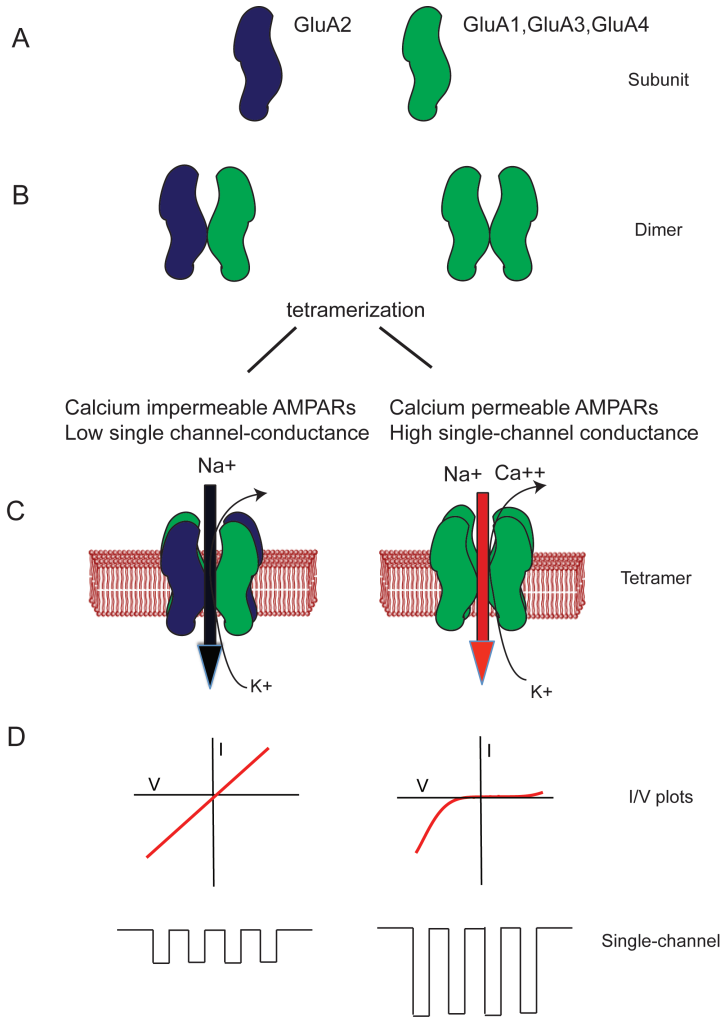


Figure 1.2. Formation of tetrameric calcium-permeable and calcium-impermeable AMPARs.

(A) GluA2 edited at the Q to R site subunit represented in blue, and GluA1, GluA3 and GluA4 represented in green. **(B)** AMPARs initially form dimers and then tetramers in the ER. The majority of AMPARs form heteromers, though homomeric receptors can also form stable AMPARs. **(C)** Heteromeric AMPARs that incorporate GluA2 are rendered impermeable to calcium, in contrast to tetrameric assemblies containing GluA1, GluA3 and GluA4, which are calcium-permeable. **(D)** Whole-cell glutamate I/V plots are linear when cells express GluA2/GluA4. In contrast, GluA1/GluA4 containing AMPARs have an inwardly rectifying I/V relationship and a larger single channel conductance. **Figure adapted from (Cull-Candy et al., 2006).**

There is substantial glycosylation and phosphorylation of AMPARs in the trans-Golgi network (TGN). Mature synaptic AMPARs are heavily glycosylated, a property, which is thought to prevent degradation of receptors (Breese and Leonard, 1993; Hullebroeck and Hampson, 1992) Indeed, a lack of glycosylation of GluA3 results in degradation of the receptor and has been linked to Rasmussen's encephalitis (Gahring et al., 2001).

The phosphorylation of AMPARs is thought to be controlled by various kinases including Ca^{2+}/calmodulin-dependent kinase II (CamKII), protein kinase A (PKA) and protein kinase C (PKC), all of which have been linked to AMPA plasticity during LTP (Barria et al., 1997; Mammen et al., 1997; Nayak et al., 1998). Indeed, Esteban and colleagues have demonstrated that PKA can control synaptic plasticity by controlling the phosphorylation of GluA4 at Ser842 and of GluA1 at Ser845 and furthermore the phosphorylation of both of these subunits during hippocampal LTP (Esteban et al., 2003). Further studies have linked the increased phosphorylation of GluA2 at Ser880 in Purkinje cells during cerebellar LTD (Chung et al., 2003).

1.1.4. AMPAR binding proteins

Synaptic AMPARs are regulated by interactions with PDZ binding proteins. These interactions have been identified using yeast-two hybrid screens carried out on the C-terminus of AMPARs. Experiments reveal a number of interacting proteins, including PICK-1, GRIP, NSF and SAP-97 (Dong et al., 1997; Srivastava et al., 1998; Wyszynski et al., 1998; Xia et al., 1999). These PDZ proteins are classified into groups depending on the identity of the last 4 amino acids in the C-terminal peptide.

The class II PDZ binding proteins include PICK-1, GRIP and SAP-97,

which recognise the X-f-X-f sequence (X=unspecified amino acid and f = hydrophobic amino acid). Unedited and edited GluA2 short and GluA3 interact with the PDZ domain containing the proteins ABP/GRIP and PICK-1, while GluA1 interacts via its C-terminus with SAP-97 and with cytoskeleton protein 4.1 homologs (Leonard et al., 1998; Shen et al., 2000). PICK-1, GRIP and NSF have been shown to control the trafficking of AMPAR from the plasma membrane after synaptic activation. Phosphorylation of GRIP has been shown to disrupt its binding to AMPARs, thus promoting internalisation of GluA2/GluA3 (Kim et al., 2001; Osten et al., 2000; Seidenman et al., 2003).

Studies have also shown that PKC phosphorylates GluA2 at Ser880 after synaptic activation of hippocampal neurons, resulting in the binding of PICK-1 to GluA2 and initiating internalisation of the receptors (Hanley, 2008). These findings were strengthened by overexpression of PICK-1 in the hippocampus, which resulted in decreased levels of synaptic GluA2 (Dev et al., 1999; Terashima et al., 2004). Furthermore, the PDZ protein SAP97 has been shown to interact with GluA1-containing AMPARs in neurons and has been shown to increase both basal phosphorylation of GluA1 at Ser845 and its density at the synapse (Tavalin et al., 2002).

1.1.5. AMPAR subunits and protein partners involved in LTP and LTD

AMPARs present at the cell surface of neurons are highly dynamic and can be rapidly internalised and re-inserted into the synapse. The dynamic regulation of AMPARs has been shown to underlie some forms of plasticity during LTP and LTD. Several early studies have shown that during the induction of LTD, AMPARs are internalised (AMPAR containing GluA2 and GluA1), resulting in a loss of AMPAR density at the synapse whilst during LTP increased insertion of calcium-permeable GluA1-containing AMPAR in

hippocampal synapses occurs (Beattie et al., 2000; Man et al., 2000; Plant et al., 2006) Investigations of AMPAR during plasticity have revealed that AMPARs undergo rapid internalisation from the cell surface via association with clathrin and AP2, leading to the formation of clathrin-coated vesicles (CCVs) (Lee et al., 2002). Internalised AMPARs are then sorted to the early endosomes, from which they are sent either to the recycling endosomes for subsequent reinsertion into the plasma membrane, or to the late endosomes. AMPARs that are contained in the late endosomes are usually degraded by lysosomes (Ehlers, 2000) (**Figure 1.3**).

Hippocampal LTD can be induced by brief application of NMDA, which has been shown to induce the rapid internalisation of synaptic AMPAR. This form of 'chemically-induced' LTD promotes increased co-localisation of AMPARs with the early endosome marker EEA1 (Ehlers, 2000). The GluA2 binding proteins PICK-1, NSF and GRIP control the internalisation of AMPARs during LTD (Perez et al., 2001). In studies where hippocampal neurons were transfected with a GluA2 mutant that lacked the NSF binding domain for GluA2, the receptor localised to lysosomes, suggesting that NSF-GluA2 interaction is required for the recycling of GluA2 to the plasma membrane. Blocking the interaction between GluA2 and GRIP or its close relative ABP inhibits LTD, suggesting that removal of surface GluA2 requires GRIP (Matsuda et al., 2000). PICK-1 has been shown to regulate the internalisation and the recycling of AMPARs to the plasma membrane. Using pH-sensitive tagged GluA2, Lin et al. (2007) demonstrated that receptors recycled more rapidly to the plasma membrane after chemical LTD in the absence of PICK-1 (Lin and Huganir, 2007).

LTP has been associated with increased trafficking of GluA1 and unedited GluA2Q to the cell surface during hippocampal LTP. Previous studies have

shown that GluA1-containing AMPARs are trafficked from the recycling endosomes where they are inserted in the cell surface of hippocampal neurons following LTP (Park et al., 2004; Plant et al., 2006). Understanding the mechanisms that control AMPAR trafficking are important as in ischemia there is substantial oxygen glucose depravation leading to increased cell-surface expression CP-AMPARs in hippocampus resulting in the increased influx of Ca^{2+} and associated cell-death (Liu et al., 2006).

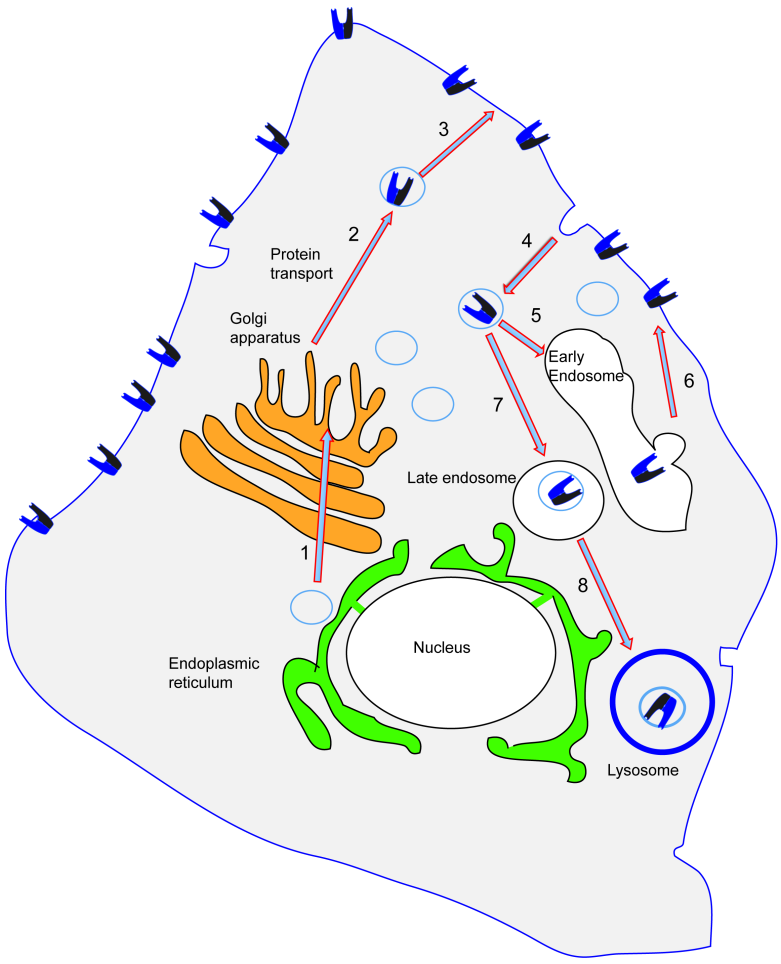


Figure 1.3. AMPAR trafficking and sorting.

(1) In the endoplasmic reticulum, AMPARs are synthesised, assembled and targeted to the trans-Golgi network where they undergo extensive post-translational modifications, including glycosylation and palmitoylation. **(2)** Mature AMPARs are trafficked from the tans-Golgi network in vesicles to the cytoplasm where they can either be stored **(3)** or trafficked to the cell surface membrane. At the cell surface membrane, AMPARs associate with proteins including PSD-95 and SAP-97. **(4)** GluA2-containing AMPARs have been shown to undergo internalisation after treatment with NMDA. Internalisation is dependent on NSF, GRIP and PICK-1, as well as the binding of the protein AP2 to the C-terminus of GluA2, leading to the formation of clathrin-coated vesicles. **(5)** After internalisation, AMPARs are trafficked to the early endosomes where the receptors can either be directed to the recycling compartment **(6)** and sent back to the plasma membrane, or they are sent to the late endosomes and finally to lysosomes for degradation (**8**). **Image modified from (Hanley, 2010).**

1.1.6. Transmembrane AMPAR regulatory proteins (TARPs)

Transmembrane AMPAR regulatory proteins (TARPs) are auxiliary proteins that control properties of AMPARs at excitatory synapses. TARPs were discovered in the mutant mouse 'stargazer'. Initially, stargazer mice were thought to have a calcium channel mutation, but further examination revealed a lack of synaptic AMPARs in cerebellar granule cells. Subsequent experiments on these mice identified a mutation in the C-terminus of the TARP Stargazin (Chen et al., 2000). In this section, I will describe the role of TARPs in controlling the properties of AMPARs.

1.1.6.1. The TARP family of proteins

Stargazin (γ-2) was the first TARP to be identified from a family of related proteins (γ-1, γ-3, γ-4, γ-5, γ-6, γ-7 and γ-8). Only five of these proteins are known to be functionally active (γ-2, γ-3, γ-4, γ-7 and γ-8). The roles of the

TARP-related proteins γ-1 and γ-6 remain unknown. *In-situ* hybridisation studies have identified that γ-2 is highly expressed in the cerebellum and is the only TARP expressed in cerebellar granule cells (Fukaya et al., 2005).

1.1.6.2. TARPs control AMPA trafficking

γ-2 increases trafficking of GluA1 to the cell surface by facilitating the exit of the receptor from the ER (Bedoukian et al., 2008). Studies have demonstrated that γ-2 also increases activation of the unfolded protein response, which increases ER protein folding capacity (Vandenberghe et al., 2005). AMPARs leaving the ER undergo phosphorylation and glycosylation in the trans-Golgi network. The glycosylation of immature AMPARs in the trans-Golgi network is enhanced by the expression of TARPs. Furthermore, cerebellar granule cells isolated from the stargazer mice contain a higher proportion of immature, glycosylated receptors failing to reach the synapse (Tomita et al., 2003).

γ-2, γ-3, γ-4, and γ-8 have identical PDZ binding domains, which contain a TTPV amino acid motif on their C-termini. The C-terminal motif of TARPs interacts with a variety of proteins, including microtubule-associated protein 1 light chain 2 (MAP1-LC2), membrane-associated guanylate kinase (MAGI-2), neuronal isoform of protein interacting specifically with TC10 (nPIST), and synaptic scaffolding proteins postsynaptic density protein of 95 kDa (PSD-95) (Deng et al., 2006). nPIST is required for targeting AMPARs to the synapse via interaction with γ-2 in the TGN. When the AMPA-γ-2 complex is inserted into the membrane, the nPIST is released from γ-2 and recycled to the TGN (Cuadra et al., 2004) (Figure 1.4). The PDZ TTPV domain of γ-2 has been shown to bind to PSD-95 and is required for synaptic targeting and clustering of AMPARs (Cuadra *et al.,*

2004). Chen *et al*. (2000) also demonstrated the importance of the PDZ binding domain of γ-2; synaptic AMPA expression was rescued when stargazer granule cells were transfected with wild-type γ-2 protein. However, AMPAR expression was not rescued in stargazer granule cells transfected with a C-termini deletion of γ-2.

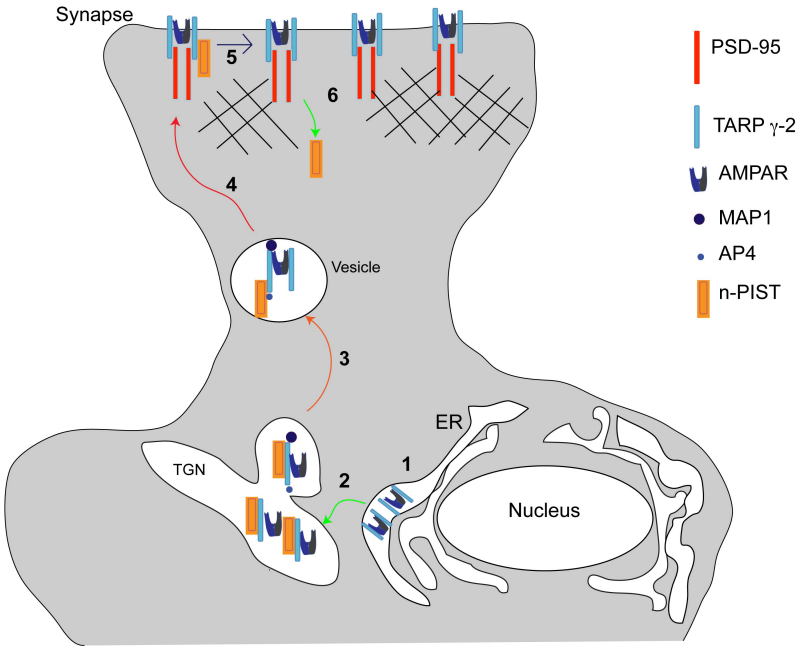


Figure 1.4. TARPs increase cell surface trafficking of AMPARs.

(1,2) AMPARs form tetramers in the ER and are exported to the Golgi. **(3)** In the Golgi, the protein nPIST associates with the C-terminus of γ-2 and, along with the proteins MAP1 and AP4, act to chaperone AMPAR to the plasma membrane. **(4)** AMPAR complexes diffuse to the postsynaptic density. **(5,6)** nPIST dissociates from the AMPA-TARP-PSD95 complex at the postsynaptic density and is recycled back to the Golgi. Figure adapted from Payne et al., 2008 (Payne, 2008).

1.1.6.3. TARPs determine AMPAR channel properties

TARPs regulate many fproperties of AMPAR channels, including receptor desensitisation, receptor deactivation and single-channel conductance. The effects of TARPs on AMPA properties have been investigated using rapid application (100 ms) of glutamate onto outside-out patches from HEK

cells that co-express AMPARs and γ-2, γ-3, γ-4, γ-7 and γ-8 (Figure 1.5a-c). These studies showed that TARPs significantly increase the AMPAR single-channel conductance, whilst having no detectable effect on the peak open probability ($P_{o,\ peak}$). Furthermore, while all TARPs slow the desensitisation of AMPARs, γ-4 and γ-8 have the most dramatic effect on AMPAR desensitisation kinetics. Further investigation of the hippocampal AMPA receptor kinetics of γ-8 knockout mice revealed faster decay kinetics compared to wild-type controls (Rouach et al., 2005).

TARPs have also been shown to modify the affinity of the AMPAR agonist kainate for this receptor. In studies of outside-out patches from GluA1-containing AMPARs exposed to kainate prior to glutamate, a small partial response to kainate was observed. However, when GluA1 was co-expressed with γ-2, kainate produced a large non-desensitising current. The increase in the peak kainate current can be measured against the peak glutamate current to produce a ratio: I_{KA}/I_{Glu}. The I_{KA}/I_{Glu} ratio is higher for AMPA co-expressed with γ-2 and γ-3 than the ratio for AMPA co-expressed with γ-4 (Shi et al., 2009). TARPs also increase the affinity of the AMPAR for glutamate (Tomita et al., 2005a). The co-expression of AMPARs with γ-2, γ-3, γ-4 and γ-8 also reduces the half-maximal effective concentrations of glutamate (EC_{50}) (Figure 1.5d) (Coombs et al., unpublished).

One of the characteristic properties of calcium-permeable AMPARs is that they are blocked by intracellular polyamines at depolarising potentials (Bowie et al., 1998; Koh et al., 1995). Calcium permeable-AMPARs, which are associated with γ-2, have reduced sensitivity to a polyamine block at positive and negative potentials. Relief of the polyamine block in CP-AMPARs when expressed with γ-2 results in decreased rectification when

compared with control cells, which lack γ-2 (Soto et al., 2007) (Figure 1.5e).

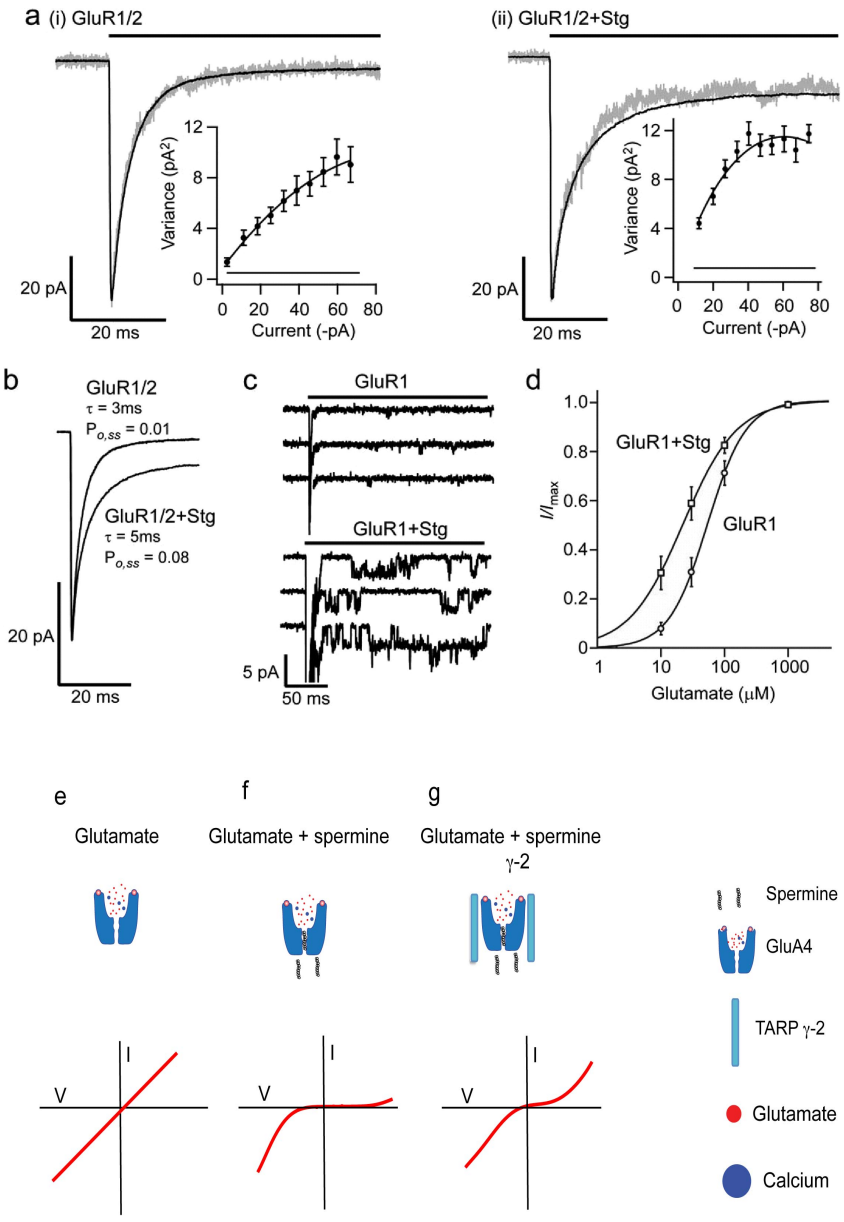


Figure 1.5. TARPs control properties of AMPARs.

(a) The properties of heteromeric GluA1/GluA2 (GluR1/2) were examined with and without stargazin (stg), achieved using tsA201 cells transfected with AMPARs and removed outside-out patches exposed to 10 mM glutamate (100 ms) at -60 mV. Current-variance plots were obtained from non-stationary fluctuation analysis applied to patches from cells expressing GluA1/2 (control) and from cells

expressing GluA1/2+stg (stargazin). Stargazin (γ-2) significantly increased single-channel conductance compared to control values. **(b)** AMPAR traces obtained from control GluA1/2 and GluA1/2+stg expressing cells. Superimposed traces demonstrate the ability of stargazin to slow the desensitisation kinetics and increase the steady-state current. **(c)** Single-channel openings obtained from the tails of current traces from control GluA1 and GluA1+stg. Stargazin increased the single-channel conductance. **(d)** Glutamate dose-response from GluA1 control and GluA1+stg. Stargazin increased the affinity of glutamate for AMPARs. **(e-g)** CP-GluA1 receptors are blocked by intracellular polyamines at -60 mV, which results in changes to current-voltage (I/V) plots. Stargazin (γ-2) relieved the polyamine block of GluA1 receptors at positive and negative potentials, resulting in less inward rectification of the I/V response when compared to the response of GluA1 alone. **Data obtained from (Coombs and Cull-Candy, 2009) and Soto et al., 2007.**

1.1.6.4. TARPs and disease

The ability of TARPs to increase the conductance and slow the kinetics of AMPARs also influences synaptic communication (Chen et al., 2000). However, in pathological conditions, where increased influx of calcium through CP-AMPARs occurs, AMPARs can also influence neuronal cell damage. (Tomita et al., 2007) used γ-8 knockout mice to study the effect of TARPs on kainate toxicity and neuronal cell damage in the hippocampus. Kainate-induced damage of neurons in the hippocampus of γ-8 knockout mice was significantly reduced, possibly due to a reduced number of AMPARs on the cell surface and reduced AMPAR channel conductance, resulting in faster channel kinetics.

Recent studies have further demonstrated that there is a correlation between mutations in the gene that encodes γ-2 with hearing loss, epilepsy and schizophrenia. These studies highlight the importance of TARPs in

controlling the properties of AMPARs and the potential for TARPs as therapeutic targets for neurological disorders (Kato et al., 2010).

1.2. Metabotropic glutamate receptors

mGluRs are G-protein coupled receptors (GPCRs) that are compromised of eight different subtypes. Group I mGluRs, (mGluR1 and mGluR5) can couple to Gq, which activates phospholipase C (PLC) and leads to an increase in intracellular calcium (Conn and Pin, 1997). Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8) receptors are linked to the inhibitory G_i/G_o, which is negatively linked to adenylyl cyclase (AC) when expressed in expression systems (Hermans and Challiss, 2001). mGluRs are known to be involved in the induction of LTD in the cerebellar cortex. mGluR regulation of LTD in this brain region is dependent on the activation of mGluRs on Purkinje cell (PC) dendrites (Kano and Kato, 1987). Subsequent studies revealed that mGluR activation could induce LTD in several other brain regions, including the neocortex, hippocampus, midbrain and striatum (Feldman, 2009; Ito, 1996; Luscher and Huber, 2010).

In the following section, I will briefly describe the role of group I mGluR in synaptic plasticity and communication in specific brain regions.

1.2.1. mGluR-coupled intracellular secondary messenger systems and intracellular pathways

mGluR1 and mGluR5 receptors are often linked to the Gq second messenger systems, which are sensitive to pertussis toxin (PTX) (Aramori and Nakanishi, 1992; Houamed et al., 1991; Prezeau et al., 1992). The activation of either Group I subtype triggers the cleavage of phosphatidylinositol 4,5 bisphosphate into diacylglycerol (DAG) and

inositol 1,4,5-trisphosphate (IP_3) by PLC, which leads to increased release of intracellular calcium by activation of the IP_3 and ryanodine receptors present on the surface of the ER (Verkhratsky, 2002) (**Figure 1.6 pathway 3**).

Activation of mGluR1 has been linked to increased protein synthesis by activating PI3K and Akt, both of which are part of the mTOR pathway in mammalian cells (Banko et al., 2006; Hou and Klann, 2004). mGluR1 and mGluR5 are directly coupled to the Homer proteins 1a, 1b, 1c, 2, and 3 (Brakeman et al., 1997). The mGluR-Homer-PI3K pathway leads to activation of 5'TOP-containing mRNA, thereby enhancing protein translation (Ronesi and Huber, 2008) **(Figure 1.6 pathway 1)**. Homer proteins are highly enriched at glutamatergic synapses and interact with PI3K enhancer, which has been linked to neuroprotection. When activated, this process can prevent apoptosis (Rong et al., 2003). The activation of group I mGluRs has been linked to the activation of the MAPK/MEK-JNK pathway by the protein Rap1 (Cavalli et al., 2001). Furthermore, the JNK protein kinases are activated by the ERK pathway, which is activated after the binding of glutamate to mGluR1 {Li, 2007 #5873; Huang et al., 2004).

Activation of mGluRs is known to modify the properties of receptors, including $GABA_A$, certain K+ channels, AMPARs and voltage-gated calcium channels (VGCC). In cerebellar granule cells, tandem pore potassium channels (TASKs) are inhibited by activation of mGluR1 and mGluR5 (Chemin et al., 2003). In cerebellar PCs and hippocampus the stimulation of mGluRs modifies properties of GABAergic currents, and in retinal amacrine cells activation of group I mGluRs have been shown to influence the activation of the calcium-calmodulin pathway, which in turn inhibits $GABA_A$ receptors (Hoffpauir and Gleason, 2002; Llano and Marty,

1995; Poncer et al., 1995). The activation of mGluR1 in cerebellar PCs results in PKC-dependent phosphorylation of the C-terminus of GluA2 Ser880 and the release of GRIP, allowing endocytosis of AMPARs from the cell surface (Chung et al., 2003). By contrast, the activation of mGluR1 in CA1 pyramidal neurons triggers the internalisation of AMPARs in a PKC-independent manner, which in this instance is regulated by the dephosphorylation of tyrosine residues on the C-terminus of GluA2 (Gladding et al., 2009).

Recent studies have linked mGluR1-mediated internalisation of AMPARs to the enzymatic activity of TACE, which cleaves Aβ amyloid and is involved in Alzheimer's disease (AD). There is also evidence that hippocampal neurons transfected with Aβ1-42 (a mutant AD peptide) have significantly reduced lateral mobility in of mGluR5 in the membrane. This deficit in mGluR mobility could be linked to the cognitive deficits that occur in AD (Renner et al., 2010).

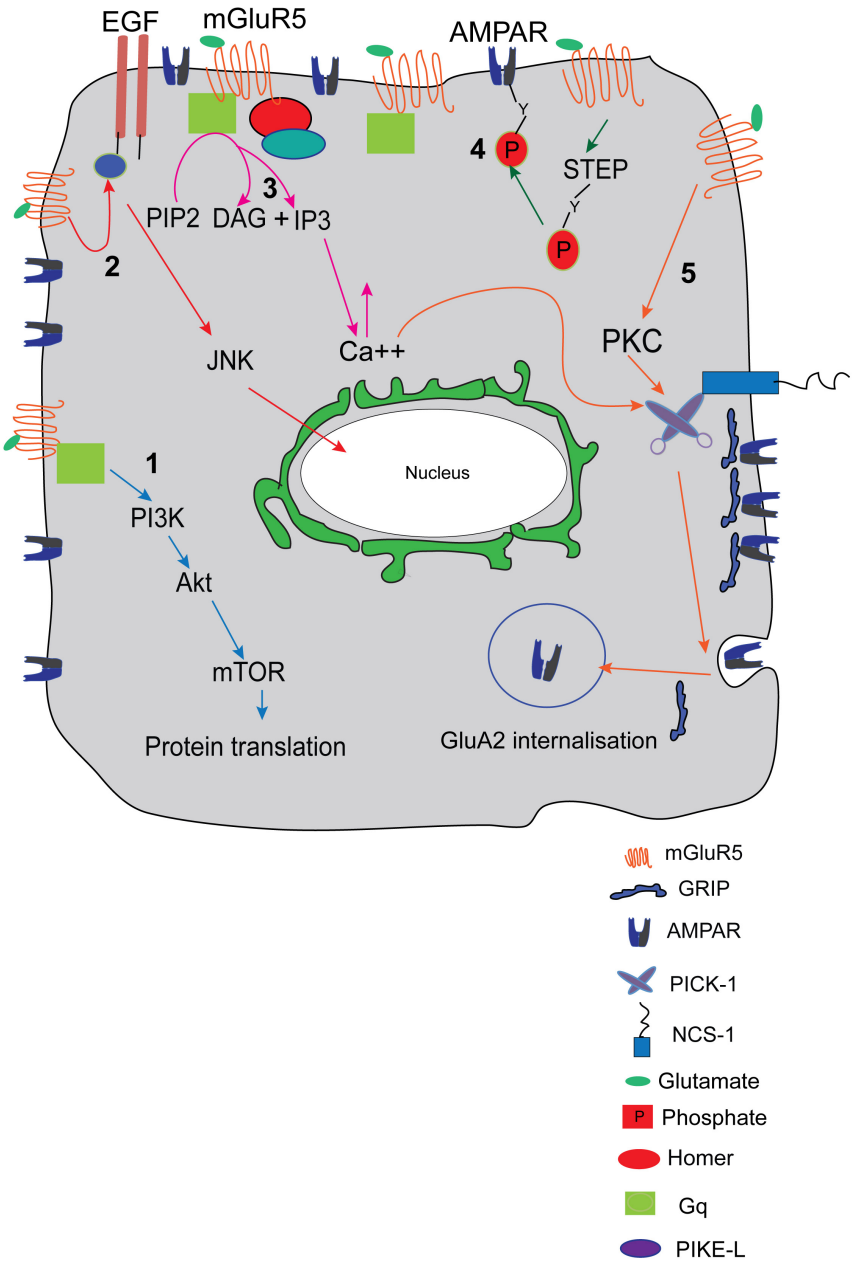


Figure 1.6. Model of mGluR1 and mGluR5 activated intracellular pathways.

(1) mGluR1 and mGluR5 activation initiates an increase in protein synthesis via the PI3K-Akt-mTOR pathway. This activation can be inhibited by cyclohexamide (C-hex). **(2)** mGluR5 stimulation leads to activation of the epidermal growth factor kinase (RTK), triggering activation of the downstream kinase JNK and initiating transcription. **(3)** Binding of glutamate to mGluR1 and mGluR5 leads to the cleaving of phosphatidylinositol 4,5- bisphosphate into diacylglycerol (DAG)

inositol 1,4,5-trisphosphate (IP_3) by PLC. This cleaving leads to the release of intracellular calcium through the IP3 and ryanodine receptors present on the cell surface of the ER. **(4)** mGluR1 activation leads to the phosphorylation of STEP and dephosphorylates the tyrosine residue to the C-terminus of GluA2, triggering internalisation (**5**) mGluR5 stimulation leads to increased release of intracellular Ca^{2+} causing association of the calcium-sensing protein (NSC-1), PICK-1 and PKC. This association results in phosphorylation of GluA2, which in turn triggers endocytosis and removal from the synapse during LTD. **Illustration adapted from Jo et al., 2008, Ferraguti et al., 2008** (Jo et al., 2008) (Ferraguti et al., 2008).

1.3. Purinergic receptors

Like glutamate, adenosine triphosphate (ATP) is also a major neurotransmitter in the CNS. ATP activates purinergic receptors, which have been shown to regulate hippocampal synaptic plasticity (Wang et al., 2004). Purinergic receptors are divided into two classes, together consisting of eight family members of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_3$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$): ion channels (P2X) and GPCR-linked (P2Y receptors) (Abbracchio et al., 2009; Fredholm et al., 1994). P2Y receptors, are distributed within the CNS, but are also found in non neuronal cells where they regulate platelet aggregation and blood clotting (Moers et al., 2003).

P2Y metabotropic purinoreceptors have a structure similar to mGluRs, as both of are 7-transmembrane domain G-protein coupled receptors. P2Y purinoreceptors, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ $P2Y_{11}$ and $P2Y_{14}$ receptors are coupled to the excitatory Gq and have the ability to form heteromeric assemblies, which are present in many cell types (Fields and Burnstock, 2006). ATP binds to the $P2Y_1$ receptors, triggering formation of DAG and

IP_3 and eliciting the release of Ca^{2+} from the ER. In contrast $P2Y_{12}$ and $P2Y_{13}$ are negatively linked to adenylyl cyclase.

The $P2Y_1$ and $P2Y_2$ receptors are present in a variety of brain regions, including the hippocampus, hypothalamus and prefrontal cortex. $P2Y_4$ and $P2Y_6$ are present in the cerebellum and hippocampus. ATP application has been shown to activate inward current responses from dorsal horn neurons in the spinal cord (Jahr and Jessell, 1983). Subsequent studies on brain slices demonstrated the presence of miniature synaptic currents, which were blocked with the competitive purinergic receptor antagonist suramin (Edwards et al., 1992). This study suggests that ATP was able to act as a neurotransmitter in the CNS. mGluR and P2Y receptors are highly localised in several glial cell types and have important roles in neuronal-glial communication, which will be discussed in the next section.

1.4. Glial cells

Glial cells are categorised into astrocytes, microglia and oligodendrocytes. Astrocytes include cerebellar Bergmann glia, which ensheathe PCs and express CP-AMPARs (GluA1, GluA4) that are activated by glutamate released from climbing fibres (CF) and parallel fibres (PF) onto purkinje cells (PC) synapses. The release of glutamate activates CP-AMPARs on Bergmann glia cells (BGCs) are important for mediating for neuronal-glial communication.

Oligodendrocyte precursor cells (OPCs) are widely distributed in the developing CNS and form myelinating oligodendrocytes. Several studies have revealed that OPCs express a mixture of calcium permeable and calcium impermeable AMPARs (GluA2, GluA3, GluA4), studies have also shown that OPCs can form synapses with hippocampal neurons and can

undergo AMPAR plasticity after neuronal activation (Bergles et al., 2000; Ge et al., 2006).

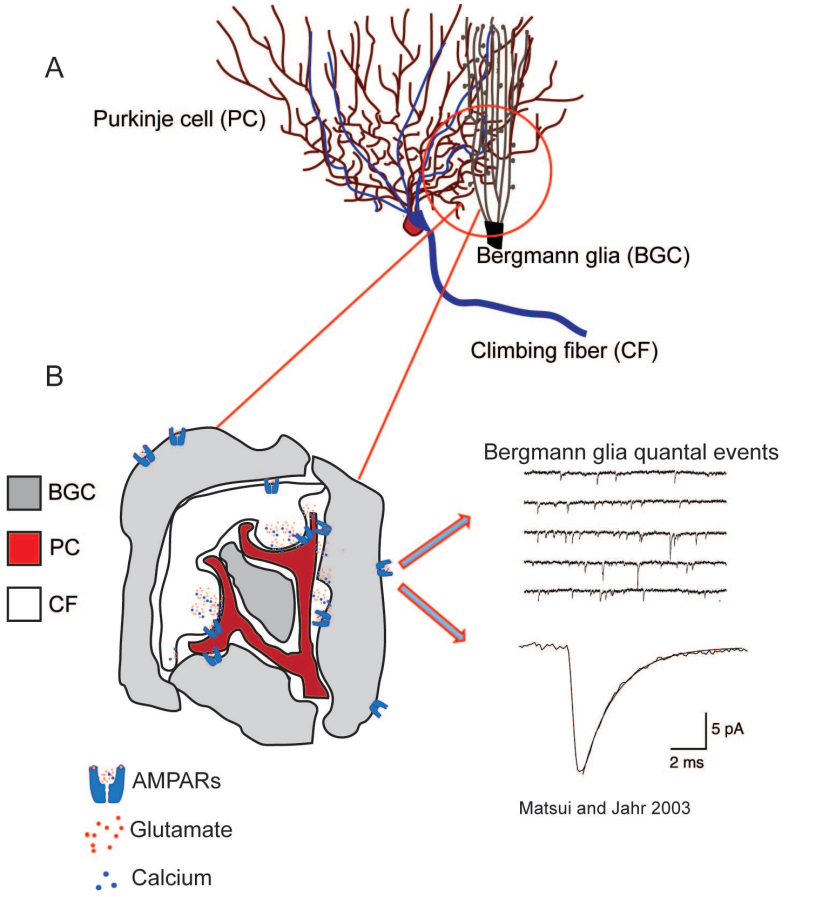
In this section I will be briefly describing the role of Bergmann glia CP-AMPARs for neuronal-glial communication. I will also be summarising the role of oligodendrocyte precursor cells in the CNS.

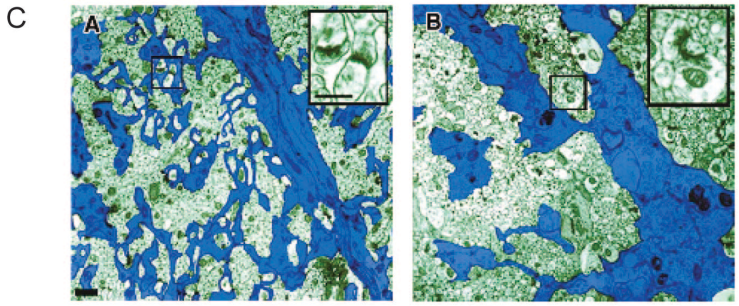
1.4.1. Bergmann glia

BGCs are the major radial glial cell of the cerebellum and have an important role in the axon guidance of Purkinje cells (PCs) and granule cells during the development of this region (Ango et al., 2008). BGCs also ensheathe PF and CF synapses. BGC currents produced by the release of glutamate induced by the stimulation of CFs and PFs have been shown to be generated by the activation of calcium permeable AMPARs and electrogenic glutamate transporters (Bergles et al., 1997; Clark and Barbour, 1997) (**Figure 1.7A**). BGCs express a high density of the glutamate transporters GLAST and GTL-1, which have important roles in regulating the levels of extracellular glutamate released after the stimulation of the granule cell layer and parallel fibres (Bergles et al., 1997; Takayasu et al., 2006). CP-AMPARs (GluA1 and GluA4) are activated by the release of glutamate from PF and CF synapses. (Iino et al., 2001) highlighted the importance of CP-AMPARs for the ensheathment of PCs. Overexpressing GluA1 in BGCs resulted in maintenance of the characteristic rod-like structure, with lateral appendages radiating from the shaft **(Figure 1.7C(a))**. However, BGCs virally transfected with GluA2 had significant retraction of lateral appendages and less ensheathment of PCs, in addition to reduced clearance of glutamate **(Figure 1.7C(b))**. The normal activation of BGC AMPARs is known to arise from spillover of

transmitter from CF-PC synapses (Bergles et al., 1997) and from quantal release originating from CFs and PFs (Matsui et al., 2005) (**Figure 1.7B**).

The release of glutamate from PCs has also been shown to induce short-term plasticity of extrasynaptic AMPARs in BGCs (Bellamy and Ogden, 2005). In this study, the extrasynaptic currents (ESCs) were recorded from BGCs in response to two stimulation pulses of the PFs, which were separated by 10, 100 and 500 ms at a baseline frequency of 0.033 Hz (**Figure 1.7D**). The stimulation of the PFs produced a 4.2-fold facilitation of the BGCs extrasynaptic responses, known as paired pulse facilitation (PPF) that was blocked by the AMPA antagonist NBQX. The tetanic stimulation of PFs, for 10 pulses at either 10 Hz or 100 Hz, also initially facilitated the BGC ESCs, though this was followed by rapid depression in the amplitude of the BGC responses. Following this study, (Bellamy and Ogden, 2006) demonstrated depression of the BGC ESC AMPAR component and uptake currents after repetitive stimulation of the PFs.


A
Purkinje cell (PC)
Bergmann glia (BGC)
Climbing fiber (CF)
B
BGC
PC
CF
Bergmann glia quantal events
5 pA
2 ms
AMPARs
Glutamate
Calcium
Matsui and Jahr 2003


C
A
B

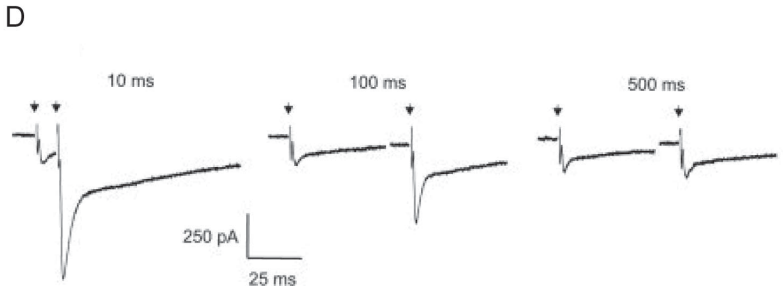

D
10 ms
100 ms
500 ms
250 pA
25 ms

Figure 1.7. CF-PF synapses release transmitter onto ensheathing Bergmann glia cell processes.

(A-B) Cerebellar climbing fibres make synaptic connections with Purkinje cells (PCs), which are encapsulated by Bergmann glial cells. Bergmann glia express CP-AMPARs that are activated through ectopic release of glutamate from Purkinje cells (**C**) Expression of calcium-impermeable AMPARs (GluA1) in BGCs alters the ensheathment of CF-PC synapses. Images (left) from control BGCs (blue) transfected with calcium-permeable AMPARs have normal connectivity with synapses. BGCs transfected with calcium-impermeable GluA2 (blue) have retracted processes. **(D)** Traces of paired-pulse facilitation (PPF) of BCG extrasynaptic currents (ESC). The ESCs were recorded in response to two stimulation pulses of parallel fibres, which were separated by varying durations (10, 100 and 500 ms). **Panel A adapted from (Beierlein and Regehr, 2005), Figure B taken from (Matsui et al., 2005), image C taken from (Iino et al., 2001) and figure D taken from (Bellamy and Ogden 2005).**

1.4.2. Oligodendrocyte precursor cells

Oligodendrocyte precursor cells (OPCs) form a major component of the glial cells in the mammalian developing central nervous system (CNS), however thet only form 5% of the glial cell population in the adult CNS. These cells were initially isolated from the optic nerve in pioneering work by Raff and colleagues (Ffrench-Constant and Raff, 1986; Raff et al., 1983). The optic nerve was an ideal preparation for the isolation of OPCs as it is a relatively accessible tissue containing OPCs along with no neuronal cells bodies (Barres et al., 1988). Raff et al., 1983 demonstrated that it was possible to distinguish OPCs from neurons and other glial cells present in the nerve express as OPCs express distinctive O-Antigens on

their cell surface. O-Antigens, which are sulfatides, are differentiation markers expressed on the surface of oligodendrocytes. O4 is an O-Antigen that is used as a marker for cell bodies and processes of OPCs (Bansal et al., 1989). Expression of O4 occurs from day 3 onwards in OPC cell cultures. While the majority of OPCs express O4, a subset of OPCs does not. This subset of OPCs expresses $NG2^{+,}$ chondroitin sulphate (Trotter et al., 2010).

It is possible to maintain OPCs in a precursor phase using two mitogens: basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF-α) (Shi et al., 1998). These mitogens both stimulate the proliferation of OPCs in culture. The removal of these mitogens and the addition of thyroid hormones to immature OPCs trigger their differentiation into oligodendrocytes (Barres et al., 1994). Oligodendrocytes begin to produce myelin basic proteins (MBP) and the O-Antigen O1, which are absent in precursor cells (**Figure 1.8**).

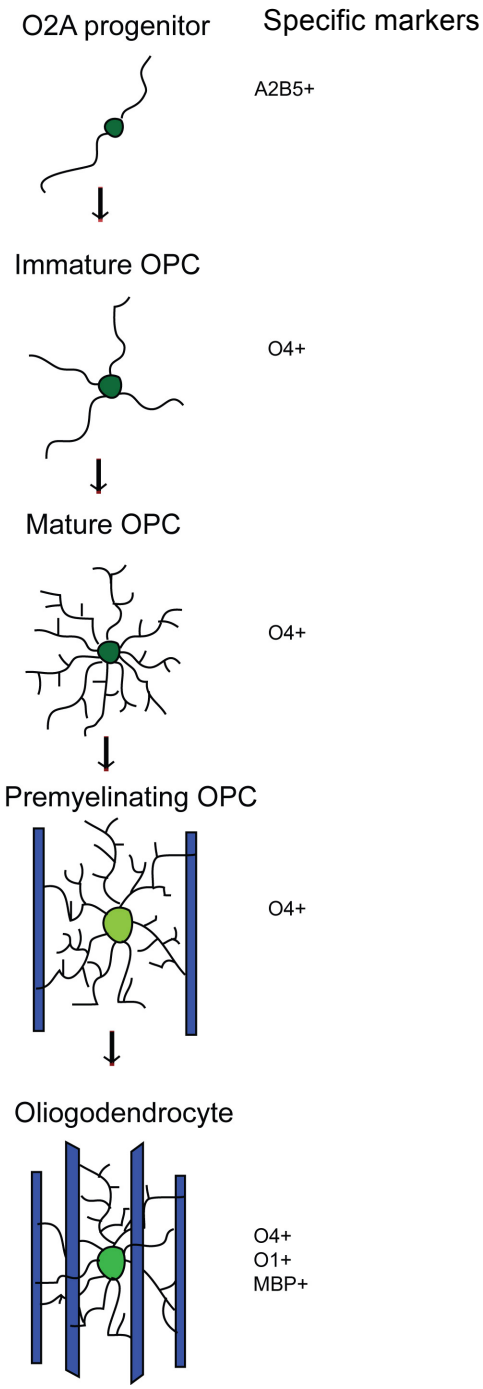


Figure 1.8. Differentiation of O2A progenitors into oligodendrocytes.

Purified perinatal progenitor cells are initially bipolar (identified with the marker A2B5). Under conditions where growth factors are present, cells will begin to differentiate and become highly branched. At this stage OPCs can be identified with the marker O4. The removal of mitogens leads to differentiation into

myelinating oligodendrocytes identifiable with the marker MBP and O1. **Figure adapted from (Levine et al., 2001).**

The ability to selectively isolate OPC in culture allowed investigations into the electrophysiological properties of OPCs, resulting in the identification of glutamate evoked responses from OPCs (Usowicz et al., 1989). Further studies examining the expression of AMPAR protein from cultured cortical OPCs, revealed the AMPA subunits present of GluA2, GluA3 and GluA4 mRNA and protein in OPCs (Itoh et al., 2002). This study also revealed that the expression level of the calcium permeable AMPARs subunits GluA3 and GluA4 are dramatically reduced in differentiated myelinating oligodendrocytes suggesting that these cells mainly express calcium impermeable GluA2. The activation of AMPARs in OPCs is required for migration, through the their interaction with integrin proteins complex (Gudz et al., 2006). In contrast there is evidence that suggests that glutamate inhibits the migration and maturation of OPCs (Yuan et al., 1998). In addition, the presence of CP-AMPARs in OPCs has been linked to an increased vulnerability of OPCs to cell death (Deng et al., 2003).

(Bergles et al., 2000) demonstrated that CP-AMPARs expressed on OPCs activated by glutamatergic inputs from CA1 neurons in the hippocampus, which were blocked in the presence of the AMPAR antagonist NBQX (Bergles et al., 2000). Ge et al*.,* (2006), then demonstrated that the release of glutamate from CA1 hippocampal neurons produced AMPAR plasticity in $NG2^{+}$ OPCs, resulting in a decrease in presence of cell surface CP-AMPARs. This study was the first to demonstrate that AMPARs on OPCs can undergo changes in response to receiving glutamate from neurons (**Figure 1.9**)**.** An increased insertion of CP-AMPARs during periods of neuronal activation is potential therapeutically important as expression of

CP-AMPARs on OPCs can increase the risk of cell death during periods of oxygen and glucose deprivation (Deng et al., 2006).

A further examination of the receptor expression profile in an OPCs cell line (CG4 cells) has shown so far that these cells express mGluRs (mGluR3 and mGluR5) (Lyut et al., 2003). Functionally activation of mGluR5 by the agonist DHPG in an OPC immortalised cell line (CG4-OPCs) has been shown to increase the levels of intracellular Ca^{2+} (Luyt et al., 2003). A2B5+ OPCs isolated from the cortex express mGluR1 expression that is a dramatically reduced in expression after two days in culture (Deng et al., 2004). However the expression levels of mGluR5 remain high for prolonged periods in cultured OPC.

OPCs have also been shown to express purinergic receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{13}$ that are activated by the neurotransmitter ATP (Fields et al., 2006). ATP is know to be released from nerve terminals and from glia and appears to have a role in neuronal-glia communication (Cotrina et al., 2000). ATP mediates synaptic transmission and is co-released with other neurotransmitters, including GABA and acetylcholine (Silinsky and Redman, 1996). Astrocytes have also been shown to release ATP, which can regulate neuronal activity (Guthrie et al., 1999).

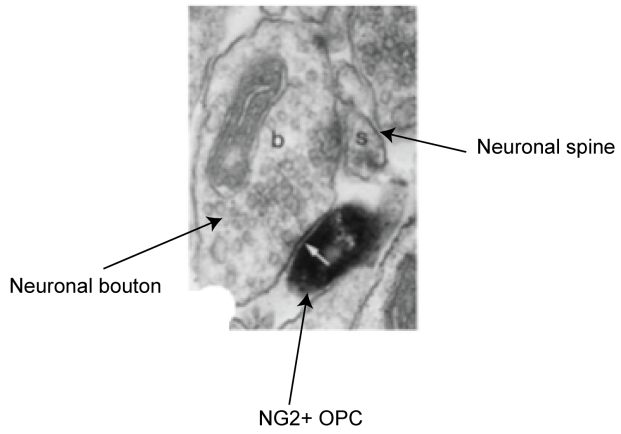
Several studies have identified the expression of purinergic receptors on astrocytes, oligodendrocytes and microglia (Table 1.11) (Fields and Burnstock, 2006). Using calcium imaging on astrocytes, ATP has been shown is important for glia-glia communication and is responsible for the propagation of Ca^{2+} waves. Utilizing genetic and pharmacological techniques it has been possible to demonstrate that multiple mechanisms lead to the release of ATP from glial cells (Wang et al., 2000). ATP also

has been shown to produce an increase in intracellular Ca^{2+} in $NG2^{+}$ OPCs, which is inhibited by the antagonists suramin and PPADS (Hamilton et al., 2010).

	Astrocytes	OPCs	Oligodendrocytes
ATP (ionotropic)			
P2X1	+	-	-
P2X2	+	-	-
P2X3	+	-	-
P2X4	+	-	-
P2X5	+	-	-
P2X6	+	-	-
P2X7	+	+	-
ATP (metabotropic)			
P2Y1	-	+	-
P2Y2	-	+	-
P2Y4	-	+	-
P2Y6		+	-
P2Y11	-	+	-
P2Y12	+	-	-
P2Y13		+	-
P2Y14	+	-	-

Table 1.11. Expression profile of P2Y and P2X receptors in astrocytes, OPCs and oligodendrocytes. Adapted from Fields and Burnstock *et al.,* 2006. (+) denotes functional evidence for the expression of ionotropic and metabotropic purinergic receptors.

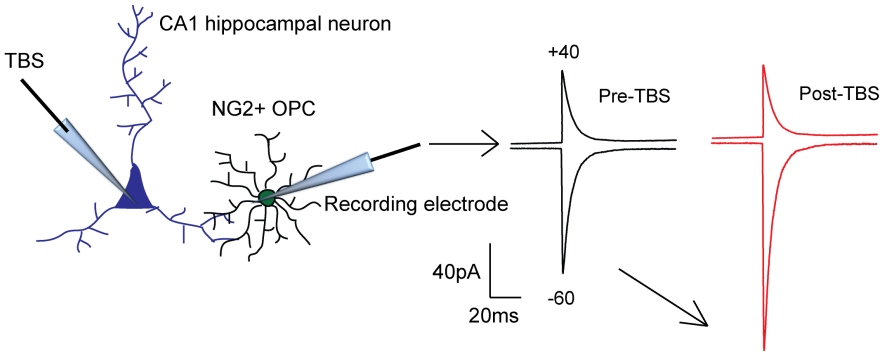
A



Bergles et al., 2000

B

C



Ge et al., 2006

Figure 1.9. Synaptic communication between neurons and OPCs in the hippocampus.

(A) Electron micrographs of biocytin-filled OPCs (black cells) which are receiving synaptic inputs from neuronal cell bouton (b) (white arrow) and a neuronal spine (s) (Bergles et al., 2000). **(B-C)** Comparison of the effects of glutamate release from neurons on the functional properties of NG2+-OPC AMPAR properties. Excitatory postsynaptic AMPAR recordings from NG2+ cells were made before and after theta burst activation of neuronal cells (10 Hz) inducing the release of glutamate. $NG2^{+}$ recordings were made at +40 and -60 in the presence of intracellular spermine. Theta burst activation of neurons results in increased amplitude of postsynaptic NG2+ AMPAR responses at +60mV. This increased amplitude of AMPAR response is indicative of an increased presence of CP-AMPARs in the cell surface of NG2+-OPCs after the release of glutamate from neurons (Ge et al., 2006). **A taken from (Bergles et al., 2000), B adapted from (Ge et al., 2006).**

2. Materials and methods

2.2.1. Cerebellum glia-neuron co-culture

All procedures were carried out in accordance with the UK Home office license (Scientific procedures) Act 1986. P7 Rats were deeply anesthetized with isoflurane and decapitated. The cerebellum was dissected from the brain and immersed in 1x Hanks balanced salt solution (HBSS) (Sigma) and maintained at 4^0C on ice. Cerebellar tissue was then minced and subsequently dissociated in 5mls of 1x HBSS containing 20 units/ml of papain containing 0.5mM EDTA and 1.65 mM L-cysteine (Worthington) for 30 minutes at 37^0C. Tissues were then triturated 10 times with a pasture pipette and centrifuged for 10 minutes at 1000 r.p.m. The pellet was resuspended in 2.4mls of papain inhibitor solution (ovomucoid protease inhibitor bovine, serum albumin and DNAase) (Worthington) and centrifuged for 10 minutes at 1000 r.p.m and plated onto 12mm coverslips coated with 100μg/ml poly-l-lysine at a density of 100 cells per coverslip. Culture media consisted of 1x Basal Medium Eagle (BME) (Sigma) containing 2mM glutamine, 10% FCS and penicillin/streptomycin (500 units/per 500mls) (Gibco). Cultures were maintained at 37^0C in a humidified incubator gassed with 5% CO2. The cultures were fed three times a week with 1xBME + FCS for 7 days (Randall and Tsien, 1995).

2.2.2. Spinal cord glia-neuron co-culture

Spinal cords were dissected from embryonic day 17 (E17) Sprague-Dawley rats (5-8 embryos). The spinal cords were removed and placed in 1XHBSS, minced and then incubated in an enzyme solution containing

Earle's salts, 20 units/ml papain (Worthington), 0.5mM EDTA and 1.65 mM L-cysteine (Sigma) for 20 min at 37°C as described above. The tissue was triturated by passages through a Pasteur pipette in complete astrocyte-conditioned media consisting of Earle's minimum essential medium (MEM) (Sigma) containing 10% fetal bovine serum, penicillin/streptomycin (500 units/each), and 20 mM glucose (Gibco). The supernatant was discarded, and the cells resuspended and plated onto polyornithine/laminin-coated (100μg/ml) glass coverslips (12 mm circular) at a density of 100 cells per coverslip. The cultures were fed three times a week with complete astrocyte-conditioned medium for 7 days (Robert et al., 2000).

2.2.3. Co-immunoprecipitation from heterologous cells

tsA201 cells were grown in 10 cm^2 dishes until a confluent monolayer was formed. Cell were then transfected with equal amounts of AMPAR and YFP-γ-5 cDNA using lipofectamine 2000 (invitrogen). After 24h of transfection, cells were washed with 1 x phospho buffered saline (Sigma) and lysed in 1ml of TNE buffer (50 mM Tris-HCl, pH, 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 plus 10μl protease inhibitor cocktail (Roche) and solubilized by rotating 1 h at 4^0C. Solubilized membranes were then centrifuged for 10 min, at 14,000g (4^0C). The pellet was discarded and the lysate membranes were placed in a new centrifuge tube. 50μl of the lysate was placed in an aliquot at denatured at 95^0C in 50μl of Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approx. 6.8.) to serve as the input protein sample. The remaining solubilized membranes were then incubated with 1 mg of rabbit anti-GFP (Invitrogen) or rabbit IgG control at 4^0C. After 24 h lysates were incubated with 20 μl of protein G bound to sepharose beads (Sigma) (1 h, 4 °C), and centrifuged for 10mins at 1300

r.p.m. The pellets were washed 2X in TNE buffer containing 300mM NaCl and once in TNE buffer. The pellets containing the bound proteins were eluted with SDS sample buffer by boiling (5 min, 95 °C). Immunoprecipitated samples and cell lysates were separated by SDS-PAGE followed by western blotting with mouse anti-GluA2 or rabbit anti-GluA1 (Chemicon; Millipore UK) and anti-GFP (Invitrogen) (Soto et al., 2009).

2.2.4. Co-immunoprecipitation from rat brain

Cerebellar tissue were removed from P20 three rats were placed in buffer A (50 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and protease inhibitor cocktail; Roche) and solubilized for 1 h at 4^0C. Tissue was centrifuged (50,000g, 45 min, 4 °C) and the lysate incubated overnight with 1 mg of antibody to γ-5 (anti–γ-5; Sigma-Aldrich). After 24 h, the lysate was incubated with 20 μl of protein G–Sepharose (Sigma; 1 h, 4 °C). The protein G pellet was washed three times in buffer A. Adherent protein was separated on an SDS gel. The proteins were transferred onto nitrocellulose and blotted with anti-SAP97 (1:100) or anti-GluA4 (1:100; both Santa Cruz Biotechnology). As internal controls, we also incubated lysates with either anti-TARP (γ-2, γ-8) antibody or anti-GluA1 (both Millipore) (Soto et al., 2009).

2.2.5. Cell surface biotinylation of AMPARs

tsA201 cells were grown in 10 cm^2 dishes and transfected with cDNA for AMPAR subunits and either GFP (as control) or γ-5. After 24 h, cells were chilled on ice and washed them twice with ice-cold PBS containing 1 mM $MgCl_2$, 2.5 mM $CaCl_2$. The cells were then treated (20 min on ice) with 1 ml of 1mg/ml of sulfo-NHS-biotin (Pierce) in 1XPBS. Un-reacted biotinylation

reagent was quenched by washing the cells three times for 5 min with 50 mM glycine in 1XPBS containing 1mM Mg^{2+} and 2.5mM $Ca^{2+,}$ followed by two washes in ice-cold 1XPBS containing Mg2+ and $Ca^{2+.}$ Cells were harvested in RIPA buffer (Perbio) and solubilized by rotating (1 h, 4 °C). Homogenates were centrifuged (14,000g, 10 min, 4 °C) and the input aliquot removed. The remaining supernatant was incubated (3 h, 4 °C) with 20 μl of 50% UltraLink Immobilized NeutrAvidin Protein (Pierce). After incubation, the NeutrAvidin protein was washed twice with high-NaCl RIPA buffer (500 mM NaCl) and once with low-NaCl RIPA (150 mM NaCl), and bound proteins were eluted with SDS sample buffer by boiling (5 min, 95 °C). Western blotting was carried out using an XCell SureLoc Novex Mini-Cell system (Invitrogen). The biotinylated proteins were probed using antibodies to GluA1, GluA2 and GluA4 (Chemicon). Immunoblots were visualized by ECL development (GE Healthcare Life Sciences) and quantified on a calibrated densitometer (Bio-Rad GS-800) (Soto et al., 2009).

2.2.6. Confocal fluorescence imaging

Fluorescence images were acquired with LEICA confocal microscope and a ×63 oil immersion lens using LSF software. Immunostaining for glia and neurons were carried out under permeabilized conditions using 0.5% Triton, in PBS. Image analysis was carried out with Image J software (public domain software developed at the US National Institutes of Health).

2.2.7. CG4 OPC cells

CG4 cells are an OPC cell line obtained from Elek Molnar (University of Bristol). The cells were used between passages 13 and 22, and were grown in chemically defined modified Sato medium containing 30% (v/v)

B104 conditioned medium. The modified Sato medium consisted of: Dulbecco's modified Eagle's medium (DMEM), 0.1% (w/v) bovine serum albumin fraction V, 60 µg/L progesterone, 16.1 mg/L putrescine, 5 µg/L sodium selenite, 400 µg/L tri-iodothyronine, 400 µg/L (T3), 50 mg/L holo-transferrin, 5 mg/L insulin and 2 mM L-glutamine. Cells were cultured in a humidified atmosphere at 37°C with 5% (v/v) CO2. Glass coverslips were coated with poly-L-lysine (100 µg/ml). 72 hrs prior to electrophysiological studies cells were treated every 24 hours with DMEM + Sato with 10ng/ml PDGF-AA and bFGF (Louis et al., 1992; Luyt et al., 2003).

2.2.8. BI04 cells

B104 cells were grown in DMEM containing 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco UK) and 2 mM L-glutamine until 70% confluent. Cells were maintained with DMEM + modified Sato medium for a period of 4 days. B104 medium used to maintain the CG-4 cells in the proliferative.

2.2.9. OPC cell purification

All procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986. Optic nerve OPC purified by immunopanning. Optic nerves were obtained from postnatal day 7 (P7) rats. Tissues were diced and digested in trypsin 0.05%/EDTA and then gently dissociated for 30 minutes at 37^0C. Dissociated P7 optic nerves were sequentially immunopanned on Thy-1 (AbD Serotec), anti-galactocerebrosidase (GalC) (Millipore), and then O4 antibody (R and D systems) plates where finally used to select GalC–O4+ OPCs. Purified OPCs to be cultured were transferred to poly-L-lysine (pLL) (Sigma) coated 24-well tissue culture plates containing proliferation medium. All cells were cultured at 37°C, 5%

CO2 in DMEM (Invitrogen) containing human progesterone (60 ng/ml), sodium selenite (40 ng/ml), N-acetyl-L-cysteine (5 µg/ml), bovine insulin (5 µg/ml) (Sigma UK), glutamine (2 mM), transferrin (100 µg/ml), bovine serum albumin (100 µg/ml), putrescine (16 µg/ml), sodium pyruvate (1 mM) and penicillin–streptomycin (100 U each) (Sigma UK), Proliferation medium also contained OPC mitogens PDGF-AA (10 ng/ml) and bFGF (10ng/ml) (both from R and D systems) (Barres et al., 1988).

2.2.10. OPC differentiation

Immature OPC cells were differentiated into pre-myelinating OPCs with the withdrawal of mitogens; basic fibroblast growth factor and platelet derived growth factor from the conditioned media. The DMEM+Sato medium was supplemented with thyroxine (T3) (Sigma T6397) for 48 hours. Cells were immunolabeled with anti-O4 to and anti-myelin basic protein to confirm adult pre-myelinating OPCs (Dugas et al., 2010).

2.2.11. Transfections

Optic nerve OPCs were grown on poly-L-lysine (PLL) glass coverslips and maintained with basic fibroblast growth factor and platelet derived growth factor for a maximum of 7 days. Cells were transfected with 2µg of DNA using the calcium phosphate transfection (Invitrogen). Cells were used for experiments 24 hours after transfections. TARP cDNAs (rat; γ-2) was a gift from Roger Nicoll (UCSF). Mutagenesis was performed using PCR, with mismatch primers obtained from Sigma Genosys (Poole, UK):

2.2.12. Immunofluorescence

Cerebellum neuronal-glial cultures grown on PLL coverslips for 10 days were washes 3X with PBS and were fixed with 4% PFA in 1X PBS for 10

minutes at 25°C. Cells were then incubated with in blocking solution (1x PBS containing 2% BSA (Sigma UK), (Sigma) 10% horse serum (Invitrogen UK) and 0.5% Triton) for 20 minutes. Coverslips were incubated with rabbit anti-γ-5 (Sigma) 1/100, mouse anti-MAP2 (Abcam) 1/50, mouse anti-EEA1 (Abcam) 1/100, mouse anti PDI rabbit anti γ-2/γ-8 (Millipore) 1/20 for 1 hour at 25^{0}C. Coverslips were finally labeled with 1/1000 rabbit anti donkey Alexa-568 and 1/1000 mouse anti-rabbit 488 (Invitrogen UK) for 1 hour at 25°C. All coverslips was mounted on anti-fade medium (Invitrogen UK).

In chapter 2 OPC cultures were grown on PLL coverslips and fixed with 4% PFA in 1X PBS for 10 minutes. Fixed cells were washed with 3x 1XPBS. Cells were then incubated with blocking solution in 1x PBS (250mg BSA (Sigma UK), (Sigma) 10% horse serum (Invitrogen UK)), and then permeabilized with 0.5% Triton (Sigma) for 20 minutes. Coverslips were incubated for 1 hour with mouse anti-O4 (1/100) (R and D systems) and with rabbit anti- γ–2/γ–8 (Millipore) (1/50) at 25^{0}C. Coverslips were washed 3x with blocking solution and finally labeled with rabbit anti donkey Alexa-568 (1/1000) and mouse anti-rabbit 488 (1/1000) (Invitrogen UK) in blocking solution for 1 hour at 25^{0}C. All coverslips were washed with 1XPBS mounted on anti-fade medium (Invitrogen UK).

2.2.13. RNA extraction

RNA was isolated from the rat optic nerve using TRIZOL (Invitrogen), and RT-PCR was carried out by using 1μg of RNA in a Retroscript kit (Invitrogen), and 5μl of the reverse transcriptase (RT). The products were amplified using the SuperTaq (Ambion) in 30 cycles of 94^{0}C(1min)/60^{0}C with 72^{0}C(7min) after the last cycle. The PCR primers were used γ-

1:Forward:GCGGGGGAAAAGAATTG**Reverse:**CAGAGCCCTGCAAAGG ;γ**2:**F:ACTACGAGGCTGACACCG**R**:ACTTAGACCTGCAGACACGAAGlγ**3 :**F:GCTGCTTAGAAGGAGCTTTCC**R**:GTTGCTTAGCCCTGCAGAG;γ**4:**F: CATCGAAGGCATCTACAAGG**R**:GATATTACTGAGGCCTGCAGC;γ**5:F**:G CTTCCTCGCAGGTGAG**R**:AAGAGAGAGGCCGGATAGGγ**6:F**:TGCCAG GAGAAGCAAACTG**R**:AGCAGCAGGCCTGAGAG;γ**7**:**F**:TTCTTTGCAGGT CGGGAG**R**:CAAGGACAGGCCTGATAAAATG;γ**8F:**CCTGGAAGGGTTGA AAAGAG**R**:TGATGTTGCTCAGGCCTG. The DNA products were resolved by using a 1% agarose gel.

2.3. Electrophysiological recordings

2.3.1. Patch electrodes

Patch electrodes were made from thick walled borosilicate capillary tubing (GC-150F; Harvard Apparatus Ltd, Edenbridge, UK) using a two-step puller (Narishige, Japan). The electrical capacitance of the electrodes were reduced by coating with Sylgard (Dow Corning 184), which was solidified using a heated coil.

2.3.2. Fast agonist application to excised patches

Outside-out patches were obtained using electrodes fabricated from borosilicate glass (1.5 mm o.d., 0.86 mm i.d.; Harvard Apparatus, Edenbridge, UK) with a resistance of 8–12 MΩ. Rapid solution switching at the patch was achieved by piezoelectric translation of an application tool. The tool was made from theta glass (2 mm o.d.; Hilgenberg GmbH, Malsfeld, Germany) pulled to a tip opening of ~200 μm, and was mounted on a piezoelectric translator (Burleigh LSS-3000/PZ-150M; EXFO Life Sciences & Industrial Division, Mississauga, Ontario or P-265.00, Physik Instrumente, Waldbronn, Germany). Control and agonist solutions flowed

continuously through the two barrels and solution exchange occurred when movement of the translator was triggered by a voltage step. To visualize the solution interface and allow measurement of solution exchange (see below) 2.5 mg/ml sucrose was added to the agonist solution and the control solution was diluted by 5%. Recorded currents were low-pass filtered at 10 kHz and digitized at 20 or 50 kHz. At the end of each experiment, the adequacy of the solution exchange was assessed by destroying the patch and measuring liquid-junction current at the open pipette; the 10–90% risetime was always <200 μs.

2.3.3. Non-stationary fluctuation analysis (NSFA)

To analyze the channel properties from macroscopic responses, glutamate (10 mM) was applied to outside-out patches (100 ms duration, 1 Hz) and the ensemble variance of all successive pairs of current responses calculated using IGOR Pro 5.05 (Wavemetrics, Lake Oswego, OR) and NeuroMatic (http://www.neuromatic.thinkrandom.com). The single-channel current (*i*), total number of channels (*N*) and maximum open probability $P_{o,\ max}$) were then measured by plotting this ensemble variance (σ^2) against mean current ($\bar{I}$) and fitting with a parabolic function:

$$\sigma^2 = iI - \bar{I}^2/N + \sigma_B^2$$

Where σ^2_B is the background variance. Along with expected peak-to-peak variation in the currents due to stochastic channel gating, some patches showed gradual changes in peak amplitude. The mean response was calculated from epochs containing 20–200 stable responses that were identified by using a Spearman rank-order correlation test (NeuroMatic). The weighted-mean single-channel conductance was calculated from the single-channel current and the holding potential (corrected for the

calculated liquid-junction potential; see above). $P_{o,max}$ was estimated by dividing the average peak current by *iN*.

2.3.4. Rectification index I/V calculation and analysis

Whole cell recordings from isolated oligodendrocyte precursor cells (OPCs) were achieved using thick-walled electrodes with a resistance 4-7 MΩ. The pipette was filled with internal recording solution containing 100 μM spermine giving a final resistance of 5-10MΩ. Input and series resistance was monitored throughout the duration of recording. A voltage ramp protocol was used to change the holding potential (–100 mV to +60 mV at a rate of 162.5 mV/s; with the voltage held at –100 mV for 200 ms). Records were filtered at 2 kHz and sampled at 5 kHz. AMPARs were activated by a bath application of 100μM glutamate. During the recordings control OPCs had an average whole cell capacitance of 10 ± 1.2pF, (whole cell compensation (30-60%) were utilized). For chapter 2 OPCs were pre treated with 100 μM DHPG for 30 minutes and then placed in the recording chamber containing external solution containing DHPG. Initially 5 control sweeps I/V recordings were obtained to determine background current without glutamate which could be an indication of voltage gated channels (**Figure 2.3.4.1**). The glutamate is then bath applied to the cells until the baseline current is stable. The rectification index **(RI)** is the determined by initially subtracting the background current from traces with agonist. The glutamate ramp IV plots were then plotted against a model cell I/V recording (+60/-100) and normalized. The rectification index was calculated by dividing the normalized value current at +60 and at -60mV. Waveforms were imported into IGOR version 6.1 (Wavemetrics, Lake Oswego, OR) and recordings were analysed using Neuromatic (http://www.neuromatic.thinkrandom.com).

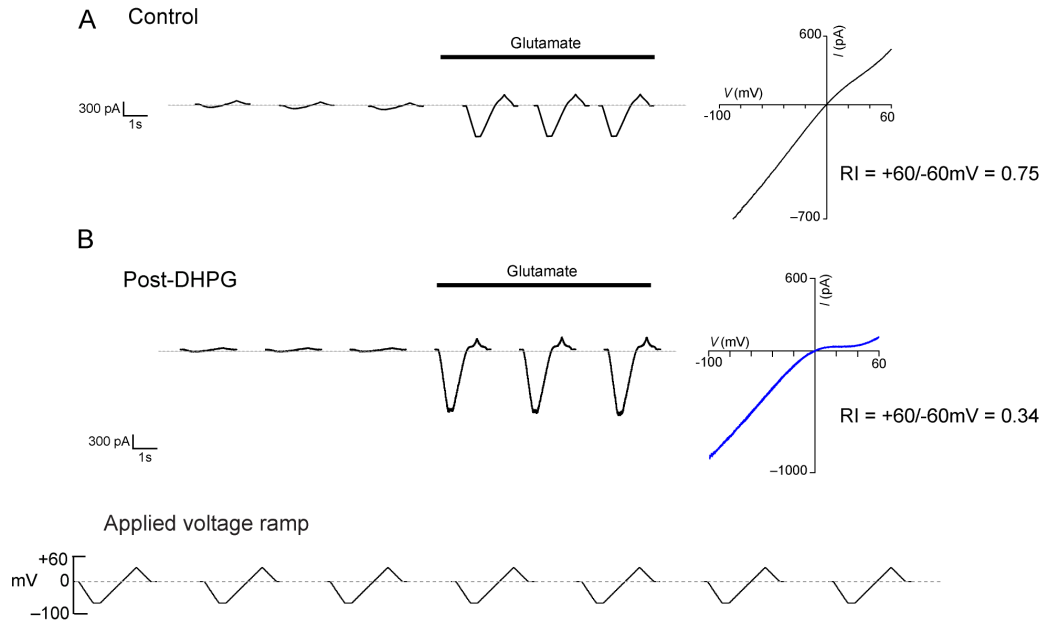


Figure 2.3.4.1. Measuring AMPA receptor rectification in CG4-OPCs in control and post-mGluR activation.

(A) Representative AMPAR responses of CG4 cells to bath applied 100μM in the presence of AP5, gabazine, TTX and strychnine. The current voltage relationship IV is obtained from the whole cell glutamate response at membrane potentials between -60 and +100. The rectification index for this cell has been calculated from current at +60mV and the extrapolated current at -100mV. **(B)** Whole cell AMPAR response to glutamate from CG4 cells treated with 100μM DHPG for 30 minutes.

2.4. Solutions and drugs

NaCl	145
KCl	2.5
$CaCl_2$	1
$MgCl_2$	1
HEPES	10
Glucose	10

External solution composed in mM. The solution pH was set to 7.3 using NaOH.

CsCl	145
NaCl	2.5
Cs-EGTA	1
Mg-ATP	4
HEPES	10

Internal solution composes in mM. The solution pH was set to 7.3 with CsOH (285± 5 mOsmol/l). Internal solution always contained 100mM spermine dihydrochloride.

2.5. Chemical names

Cyclohexamide	Inhibits protein synthesis	4-[2-(3,5-Dimethyl-2-oxo-cyclohexyl)-2-hydroxyethyl]-2, 6-piperidinedione (Tocris)
Spermine	Blocks CP-AMPARs	N,N'-Bis(3-aminopropyl)-1,4-butanediamine tetrahydrochloride (Sigma)
KN-62	Inhibits CamKII and $P2X_7$ receptors activity	-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo- 3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester (Tocris)
TTX	Na+ channel blocker	Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethan o-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol (Tocris)
DHPG	Agonist at the mGluR1 and mGluR5 receptors	(S)-3,5-Dihydroxyphenylglycine (Tocris)
Wortmannin	PI3K inhibitor	1S,6br,9aS,11R,11bR) 11-(Acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(metho

		xymethyl)-9a,11b-dimethyl-3H-furo[4,3,2-de]indeno[4,5,- h]-2-h]-2-benzopyran-3,6,9-trione (Sigma)
ACDPP	Antagonist of the mGluR5 receptor	3-Amino-6-chloro-5-dimethylamino-N-2-pyridinylpyrazinec arboxamide hydrochloride (Tocris)
MCPG	Antagonist for the Group I/II mGluR	S)-a-Methyl-4-carboxyphenylglycine (Tocris)
SP600215	Inhibits JNK activity	Anthra[1-9-cd]pyrazol-6(2H)-one (Tocris)
Strychnine	Antagonist for the glycine receptor	Strychnidin-10-one hydrochloride (Tocris)
BAPTA-AM	Calcium chelator	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (Tocris)
SR95531	Antagonist for $GABA_A$ receptors	6-Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide (Toris)
GYKI 52466	Antagonist for AMPA receptors	4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-y l)-benzenamine

		dihydrochloride (Tocris)
PhTX-433	Blocker of CP-AMPARs	tris(trifluoroacetate) salt, (*S*)-N-[4-[[3-[(3-Aminopropyl)amino]propyl]amino]butyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzenepropanamide tris(trifluoroacetate) salt (Sigma)
Kainate	Agonist for AMPA, kainate and NMDA receptors	(2S,3S,4S)-Carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid (Sigma)
ATP Magnesium	Agonist for Purinergic receptors	Adenosine 5'-triphosphate (Sigma)
RHC 80267	Inhibits Diacylglycerol (DAG) degradation	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (Tocris)
AP5	Antagonist for the NMDA receptors	D-(-)-2-Amino-5-phosphonopentanoic acid (Tocris)

2.6. Additional drugs and concentrations used

Compounds	Concentration
DHPG	100μM
BAPTA-AM	20μM
Cyclohexamide	20μM
ATP	1mM
PPADS	10μM
ACDPP	10μM
MCPG	1mM
AP5	20μM
Strychnine	1μM
Gabazine/ SR95531	20μM
TTX	1μM
Cyclothiazide	50μM
Wortmannin	100nM

2.7. Frequently used abbreviations

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
mGluR	Metabotropic glutamate receptors
CP-AMPARs	Calcium permeable AMPA receptors
RI	Rectification index
P2Y	Purinergic receptor 2Y
OPC	Oligodendrocyte

	precursor cell
TARPs	Transmembrane AMPA receptor regulatory proteins
NSFA	Non stationary fluctuation analysis
O4	O-Antigens expressed on the surface of OPCs

2.8. Statistics

All data are representative of trials repeated a minimum of 5 times across separate cell culture isolations unless otherwise indicated. Data are presented as mean±S.E.M. Multiple group differences were examined using a Kruskal-Wallis rank sum test followed by pair wise Wilcoxon rank sum tests with Holm's sequential Bonferroni correction (R 2.9.2, The R Foundation for Statistical Computing). For measuring the difference between two populations unpaired T-test with *post hoc* Tukey tests, or Mann-Whitney tests (in cases where tests of normality failed) were used to identify significant differences between treatment conditions.

Chapter 3. The novel TARP γ-5 regulates AMPA receptor trafficking and channel properties

3.1. Summary

TARPs γ-2, γ-3, γ-4, γ-7 and γ-8 have been shown to control the properties and trafficking of AMPARs in many if not all central neurons (Chen *et al*., 2000; Rouach et al., 2005). However, no TARPs have been reported that specifically regulate CP-AMPARs.

Contrary to expectation, we found that the stargazin protein family member γ-5, widely used as a negative control, act as a functional TARP and specifically regulates CP-AMPARs in Bergmann glia. The experiments described in this chapter demonstrate that, unlike other TARPs, γ-5 is highly expressed in a number of glial cell types, including those in the olfactory bulb and the spinal cord.

Furthermore, we have shown that γ-5 reduces cell surface expression of homomeric GluA2 receptors by increasing degradation of the receptor. These results suggest an important role for γ-5 in regulating the functional contribution of CP-AMPARs.

3.2. Introduction

TARPs are vital for AMPAR-mediated signalling (Milstein and Nicoll, 2008; Priel et al., 2005). The loss of γ-2 expression in mice (Stargazer) has been shown to produce a loss of AMPAR trafficking to the cell surface of granule cells in the cerebellum and ataxia in mice (Chen et al., 2000). The protein γ-5 is related to the Stargazin family of proteins. However, it does not have the ability to 'rescue' AMPAR responses in granule cells from γ-2-lacking stargazer mice. Therefore, γ-5 was not thought to be a TARP, and has been widely used as a negative control in experiments on TARPs (Tomita et al. 2003).

This chapter initially addressed the question of whether γ-5 is capable of modifying properties of CP-AMPARs. We found that γ-5 selectively regulated the functional properties of those AMPARs that contain long-form subunits, including AMPARs containing the GluA2Long (GluA2L) and homomeric GluA1 and GluA4.

Although we find that γ-5 can control AMPAR properties when co-expressed in transformed HEK293T cells (tSA201), its physiological role is more difficult to address. *In-situ* hybridisation studies have previously identified mRNA for γ-5 and the TARP γ-4 in cerebellar Bergmann glial cells (BGCs). Furthermore, γ-5 mRNA has been detected in several other brain regions, including the hippocampus, thalamus, striatum and several layers of the olfactory bulb (Fukaya et al., 2005). BGCs express only CP-AMPARs containing GluA1 and GluA4, which are activated during neuron-glia signalling. Given that γ-5 is intensely expressed in cerebellar BGCs, and that neuron–glia communication in these cells is dependent on CP-AMPARs, we have considered whether γ-5 is involved in signalling

between neurons and BGCs (Iino et al., 2003; Matsui et al., 2006; Burnashev et al., 1992).

3.3. Results

3.3.1. γ-5 regulates AMPAR channel properties

To examine whether γ-5 acts as a TARP, AMPARs were co-expressed with γ-5 in recombinant tsA201 cells. Patches were excised from the membrane of transfected cells and currents were obtained by the rapid application of 10 mM glutamate onto outside-out patches. This study initially compared responses from cells transfected with GluA4 alone with those obtained from cells expressing GluA4 + γ-5 or GluA4 co-transfected with one of the recognised TARP family members (γ-2, γ-3, γ-4, γ-7 and γ-8) (**Figure 3.1A-B**).

All previously established TARPs significantly increased AMPAR single-channel conductance (estimated from Non-stationary fluctuation analysis). TARP γ-2 increased the channel conductance of homomeric GluA4 receptors (20.1 ± 1.3 to 31.4 ± 2.5 pS; P = 0.0016, n = 8 and 9 cells, respectively). Interestingly, γ-5 also produced a marked increase in single-channel conductance of homomeric GluA4 AMPARs (from 20.1 ± 1.3 to 36.0 ± 2.6 pS; P = 0.0001, n=8 and 7 cells). The magnitude of the conductance increase seen with γ-5 was comparable to the conductance increase obtained with each of the previously established TARP family members. This unexpected finding, that γ-5 increased the single channel properties of AMPARs suggested that it is binding to, and modifying AMPARs properties (**Figure 3.1C**).

We next examined the effects of γ-5 on two AMPAR current parameters that contribute to the overall efficacy of the receptor population: the probability that agonist-bound channels will open (peak open probability, $P_{o,peak}$) and the speed of desensitisation (τ_{des}). Whereas $P_{o,peak}$ for homomeric GluA4 AMPARs was not altered by co-expression with the established TARPs, it was markedly decreased by co-expression with γ-5 (from 0.57 ± 0.03 to 0.33 ± 0.03; $P < 0.0001$, $n = 8$ and 7) (**Figure 3.1D**). However, γ-5 did not alter the desensitisation kinetics of GluA4 (3.47 ± 0.27 ms for GluA4 vs. GluA4+γ-5 3.01 ± 0.16 ms with γ-5, $P = 0.1731$, $n = 8$ and 7; compared to 5.7 ± 0.7 ms for γ-2, $P = 0.0114$, $n = 9$) (**Figure 3.1E**). This observation highlights the unique properties of γ-5 in decreasing the $P_{o,peak}$ of AMPARs without modifying the channel kinetics (3.47 ± 0.27 ms for GluA4 versus 3.27 ± 0.36 ms with γ-7; $P = 0.6525$, $n = 8$ and 5) (**Figure 3.1E**). The TARP γ-7 and γ-5 have similar protein sequence in the C-terminus and γ-7 like γ-5 also has little effect on the properties of AMPAR kinetics, suggesting that the C-terminus of TARPs are responsible for the effects on AMPARs kinetics.

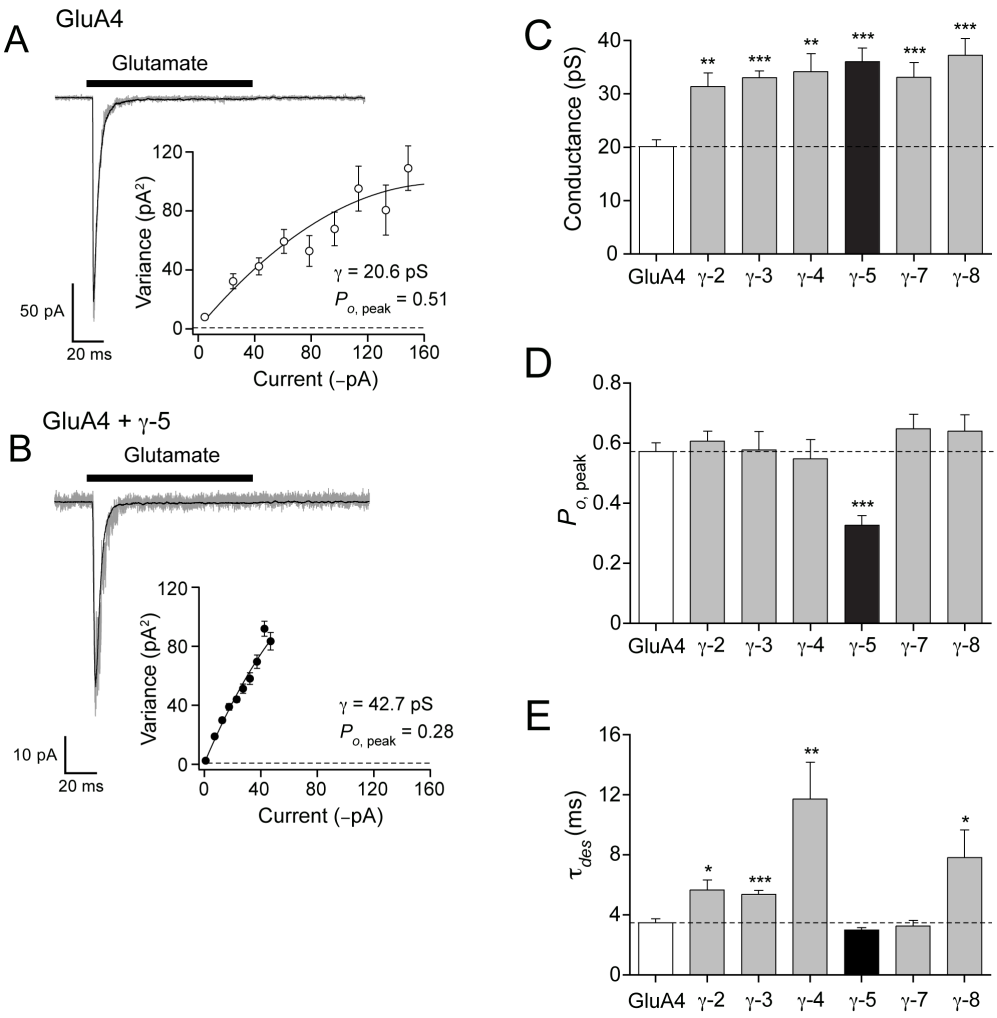


Figure 3.1. γ-5 modifies the channel properties of homomeric GluA4 AMPARs.

tsA201 cells were transfected with GluA4 and each individual member of the stargazin family of proteins (γ2, γ3, γ4, γ5, γ7 and γ8). Twenty-four hours post-transfection, the GluA4 channel properties were measured in outside-out membrane patches exposed to fast application of 10 mM glutamate applied for 100 ms (see Methods). (**A-B**) Current variance traces were obtained from tsA201 cells expressing either GluA4 alone or together with γ–5. NSFA was used to obtain conductance values for GluA4 AMPARs. (**C**) Bar graphs compare the effect of the different TARPs (γ-5, black; other TARPs, gray; no TARP, white) on the estimated mean single-channel conductance. γ-5 significantly increased the conductance of

GluA4 from 20.1 ± 1.3 to 36.0 ± 2.6 pS; *P* = 0.0001, *n*=8 and 7 patches. (**D**) The probability of agonist-bound channel opening (peak open probability, $P_{o,peak}$) GluA4 was markedly decreased by γ-5 (from 0.57 ± 0.03 to 0.33 ± 0.03; *P* = 0.0001, *n* = 8 and 7). (**E**) The time constant of the decay of receptor desensitisation (τ_{des}) for GluA4 was not increased compared with GluA4 + γ-2 - γ–8 (3.47 ± 0.27 ms for GluA4 *vs.* 3.01 ± 0.16 ms with γ-5, *P* = 0.1731, *n* = 8 and 7 cells; compared to 5.7 ± 0.7 ms for γ-2, *P* = 0.0114, *n* = 9 cells). Error bars are s.e.m. **The work for this figure was carried out in collaboration with David Soto. We each carried out equal amounts of work to produce this figure.**

3.3.2. γ-5 regulates the long-form CP-AMPARs

γ-5 was not thought to be a TARP because it failed to rescue cell surface AMPARs or synaptic responses in stargazer granule cells (Tomita *et al*., 2003). Previous studies have shown that cerebellar granule cells express predominately the short-form calcium impermeable GluA2/GluA4c subunits. The results shown in **Figure 3.1** demonstrate that γ-5 behaves like a TARP when co-expressed with a long-form CP-AMPA. Therefore, it seems possible that γ-5 does not modulate the properties of AMPARs in cerebellar granule cells either because they do not express calcium permeable subunits, or because they lack long-form AMPAR subunits. To address this issue we examined the properties of continuum of AMPARs assemblies, from homomeric GluA4 to heteromeric GluA2/4, in the absence and the presence of γ-5 expressed in tsA201 cells **(Figure 3.2A).** The same series of experiments were then repeated using AMPARs assemblies composed of entirely of long-form AMPAR subunits. This was achieved using long-form GluA2L rather that short-form GluA2s (**Figure 3.2B**).

To determine the properties of AMPARs co-expressed with γ-5, outside-out patches were excised and exposed to 10 mM glutamate and obtained a wide range of rectification values ($RI_{+60/-60}$ 0.07–1.35). It is possible to distinguish GluA2R containing AMPARs as the rectification index (RI = $+_{60mV/-60mV}$) produced were between 0.4-1.35 as polyamines do not block GluA2R containing AMPARs. However when GluA4 alone is transfected in tSA201 cells the RI ranges between 0.3-0.07 as polyamines block the pore of this CP-AMPARs at positive membrane potentials. We next plotted single-channel conductance as a function of RI, after grouping data according to whether patches showed a low (<0.4), intermediate (0.4–0.8) or high (>0.8) RI. For patches in which most receptors were expected to be homomeric GluA4 (transfected with a low proportion of GluA2R cDNA, and yielding a low RI), γ-5 produced a significant increase in single-channel conductance (from 13.4 ± 2.3 pS to 27.1±2.6 pS; *n* = 8 and 7 respectively; *P* = 0.0017) **(Figure 3.2A).** Similarly, conductance increased in patches with intermediate RI values (from 8.0 ± 0.8 pS to 15.4 ± 1.5 pS; *n* = 6 and 9; *P* = 0.0023). However, γ-5 had no significant effect on conductance for patches with a high RI (high proportion of heteromeric GluAR2/GluA4 assemblies) (8.7 ±1.1 pS with γ-5 versus 6.0 ± 1.1 pS without γ-5, *n* = 10 and 6; *P* = 0.1303). This suggested that γ-5 selectively modifies the properties of, or selectively traffic, certain CP-AMPAR assemblies. This finding was unexpected, as no other TARP seems to be able to modify or deliver specific AMPAR subtypes.

To investigate whether γ-5 is selective for long form AMPAR subunits, cells were transfected with GluA2L, which in common with GluA1 and GluA4 has a long C-terminus. The estimated single-channel conductance of high-RI patches were assessed (in which most AMPAR channels were

expected to be heteromeric GluA2 containing assemblies) producing a single-channel conductance estimates of 6.7 ± 1.0 pS (*n* = 6) in the absence of γ-5 and 14.2 ± 2.2 pS (*n* = 7; *P* = 0.0143) in the presence of γ-5. Thus, expression of γ-5 with GluA2L and GluA4 together gave a markedly increased single-channel conductance **(Figure 3.2B).** The possibility that GluA2L itself gives rise to elevated conductance in high-RI patches can be excluded, as GluA2/4 and GluA2L/4 receptors both yielded very similar conductance estimates in the absence of γ-5. Thus, the macroscopic properties of AMPARs may be modified by γ-5, irrespective of the presence of edited GluA2, provided the receptors are composed of long-form subunits.

To confirm the selectivity of γ-5 for long form AMPAR subunits rather the channels calcium permeability, we compared the effects of γ-5 on the unedited (Q) forms of GluA2(Q)s and GluA2(Q)L. As control we compared this with the effects of γ-2 on the unedited receptors. As expected, γ-2 increased the single-channel conductance of both GluA2(Q) (from 19.4 ± 1.3 to 30.5 ± 3.7 pS, *P* = 0.0044, *n* = 10 and 6) and GluA2L(Q) (from 17.7 ± 1.8 to 26.4 ± 2.3 pS, *P* = 0.0129, *n* = 7 and 6). We found that γ-5 increased the conductance of GluA2L(Q) (to 33.0±3.3 pS; *P*<0.0017, *n* = 7) but produced a significant, although less marked, increase in the conductance of the unedited short-form GluA2(Q) (26.1 ± 1.8 pS; *P* = 0.0066; *n* = 8) (**Figure 3.2C**).

We also examined the effects of γ-5 on the short-form CP-AMPAR composed of homomeric GluA3 and found that although the channel conductance of these receptors was increased, this was no less the increased conductance of the long-form AMPARs (from 21.7 ± 2.6 to 31.2

± 2.7; *n* = 9 and 12, respectively; *Ps* = 0.0183) (**Figure 3.2E-F**). It is also of note that γ-2, unlike γ-5, did not increase the $P_{o,peak}$ shown by the homomeric GluA2Q (long or short) (**Figure 3.2E).** This data supports the view that the action of γ-5 was preferentially seen with assemblies containing the long form subunits, independent of AMPAR calcium permeability.

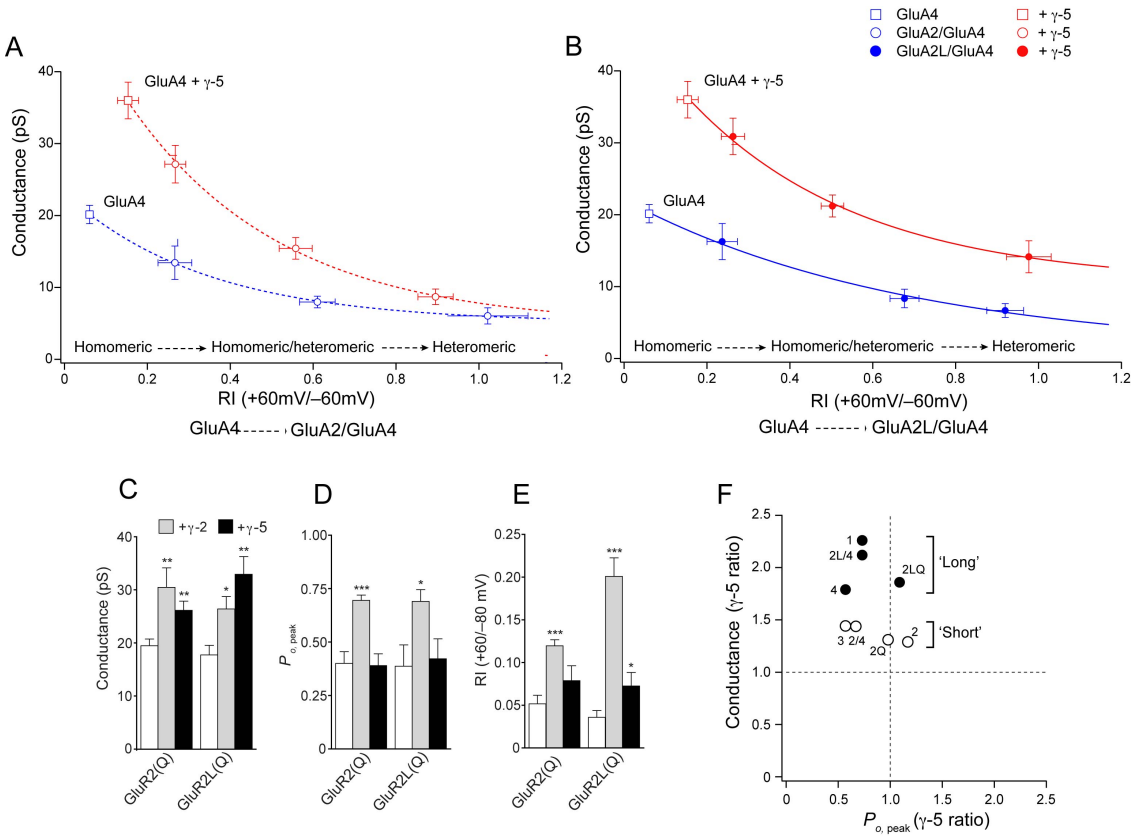


Figure 3.2. γ-5 is selective for 'long-form' AMPARs.

(A) tsA201 cells were transfected with GluA4 control, GluA4+γ-5, heteromeric GluA2+GluA4 (open circles) or GluAL2+GluA4 (filled circles) with (red) and without γ-5 (blue). Outside-out patches were exposed to 10mM glutamate for 100 ms at -60 mV. The rectification index was obtained by measuring the peak current at +60 and -60mV. NSFA was used to estimate the single-channel conductance from patches expressing a continuum of assemblies from homomeric GluA4 to heteromeric short-form GluA2+GluA4 **(A)** or heteromeric long-form GluA2L+GluA4

(B) with and without γ-5. It is of note that γ-5 increased the conductance of AMPA receptor channels when expressed with GluA2L/GluA4 (B); this contrasts with its apparent lack of effect when expressed with the GluA4/GluA2 short-form **(A).** (**C)** Single-channel conductance for both GluA2(Q) and GluA2L(Q) was increased by co-expression with γ-2 or γ-5. (**D**) Peak open probability ($P_{o,peak}$) of GluA2(Q) and GluA2L(Q) receptors was increased by co-expression with γ-2 but not γ-5. (**E**) For GluA2(Q), RI was altered by γ-2 but not by γ-5, whereas for GluA2L(Q), RI was increased both by γ-2 and γ-5. Error bars are SEM (**F)** Plots of the relationship of single-channel conductance of the long-form receptor conductance and $P_{o,peak}$ when expressed with γ-5. The long-form receptors GluA1, GluA2L(Q), GluA2L(R) + GluA4 and GluA4 conductance values were elevated when expressed with γ-5 compared to the heteromeric short-form AMPA combinations containing GluA3/GluA4 and GluA2(R)/GluA4 co-expressed with γ–5. **The work for this figure was carried out in collaboration with David Soto. We each carried out equal amounts of work to produce this figure.**

3.3.3. γ-5 regulates cell surface expression of GluA2Q

Although these results strongly support the idea that γ-5 interacts with all of the AMPAR subunits, it predominantly affects AMPARs receptors composed of long-form assemblies. Previous studies have shown that γ-2 can significantly increase the trafficking of AMPARs to the cell surface (Tomita et al., 2005). It is therefore of interest whether γ-5 might influence the trafficking of long-form AMPARs subunits. To address this question, cell surface biotinylation experiments were performed on TSA201 cells transfected with GluA1, GluA2 short (GluA2s), GluA2L, GluA3 and GluA4 expressed with YFP-γ or with GFP as a control. For loading controls membranes were probed with anti-tubulin antibody. There were no differences in the total amount of tubulin protein between GFP and γ-5 transfected GluA2 receptors. We found that γ-5 dramatically reduced the surface expression of both GluA2s(Q) and GluA2L(Q) (Cell surface control GluA2Qs (s) 66% ± 17% to 6% ± 3% for GluA2s(Q) + γ-5 (n = 5, P = 0.0259*), and cell surface control GluA2QL =82% to 45% for GluA2L

(Q)+γ-5 n = 5 P = 0.033) (**Figure 3.3C**). However, γ-5 had little effect on other AMPAR subunits examined. Therefore γ-5-induced decrease in surface expression was not solely dependent on AMPAR C-tail length.

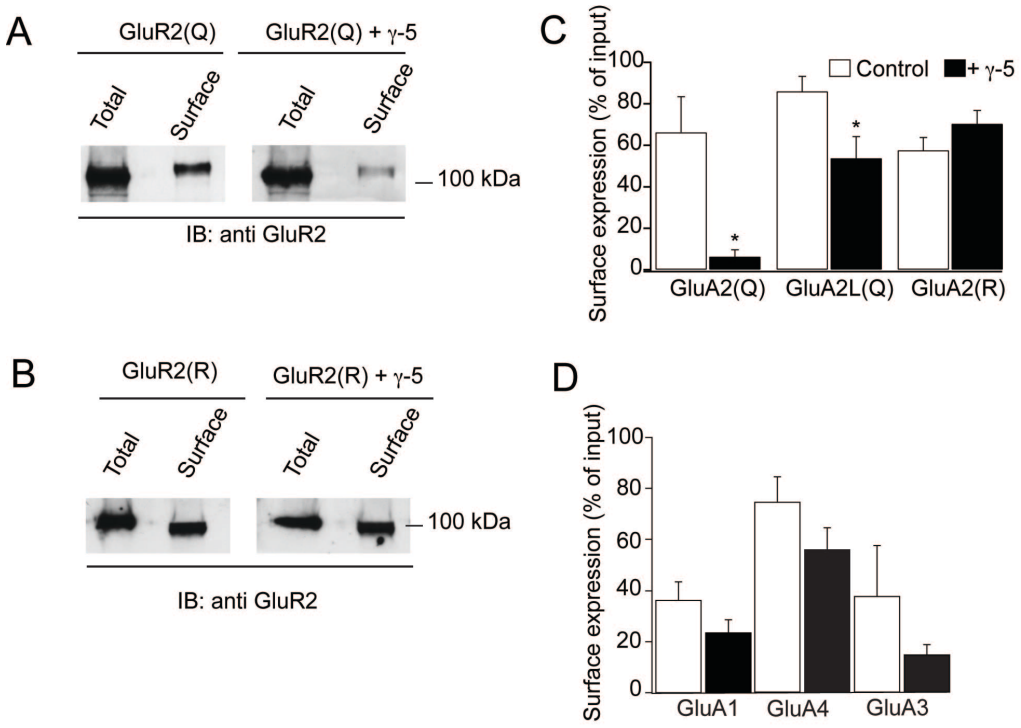


Figure 3.3. The TARP γ-5 controls AMPAR trafficking.

tsA201 cells were transfected with GluA1, GluA2(Q)short, GluA2(Q)long, GluA2(R)short, GluA2(R)long, GluA3 or GluA4. Cells expressing each of these AMPA receptor assemblies were co-transfected with either GFP (control) or γ-5. **(A)** Western blot representing the cell surface expression levels of unedited GluA2(Q) (left) compared to cell surface expression levels when co-expressed with γ-5(right). **(B)** Western blot representing cell surface expression of edited GluA2short in control conditions and co-expressed with γ-5. **(C)** Cell surface expression of GluA2 (Q) short and long receptors was significantly reduced in cells co-expressing γ-5 (GluA2(Q)+GFP = 63% percentage of cell surface; GluA2 (Q)+γ-5 = 5% cell surface), (GluA2L+GFP (Q) = 82%, GluA2L (Q)+γ-5 = 45%) (Input refers to the total amount of protein). Pooled data from the biotinylation assays for GluA2 (Q), GluA2 (Q) L and GluA2(R) (n = 5 repetitions; P = 0.0259* and P = 0.033*) **(D)** γ-5 does not effect the cell surface expression of GluA1, GluA3 and GluA4. Bars are mean average + S.E.M.

3.3.4. γ−5 protein is expressed in BGCs

γ-5 mRNA has previously been identified in BGCs from adult rat brain slices (Fukaya et al., 2005). To test the presence of the γ-5 protein expression in cultured cerebellar glial cells, cells were cultured from P5 rats and maintained for 7 days in vitro. Over 80% of cells labelled with an antibody against glial fibrillary acidic protein (GFAP), a widely used glial cell marker known to be expressed in Bergmann glia (Ango et al., 2008; Custer et al., 2006). Once the presence of GFAP expressing astrocytes were identified in culture, cells were the labelled with an antibody raised against the whole rat γ-5 protein and a secondary anti-rabbit Alexa568 marker (red). The majority of cultured cells that were labelled with γ-5 showed the fusiform morphology characteristic of cultured BGCs (Burnashev et al., 1992a) (**Figure 3.4**).

3.3.5. γ-5 co-immunoprecipitates with native AMPARs

To establish whether γ-5 was bound to native AMPAR subunits, we examined the ability of γ-5 to co-immunoprecipitate with native GluA1 and GluA4 in cerebellar cells from P20 rats n = 3. We identified that γ-5 can co-existed as a complex with GluA1 and GluA4 in cerebellar cells **(Figure 3.5 A-B)**. To determine whether γ-5 bound to all AMPAR subunits co-immunoprecipitation assays were performed from HEK 293T cells transfected with a yellow fluorescent protein (YFP)–γ-5 and AMPAR subunits. We also investigated if γ-5 bound to long and short GluA2. These experiments revealed that γ-5 protein complexes with both GluA2(Q) and GluA2L(Q) n = 3 (**Figure 3.5C**). This is consistent with the view that γ-5 interacts with all AMPAR subunits, but that it modulated predominately functional properties of the long-form AMPARs subunits.

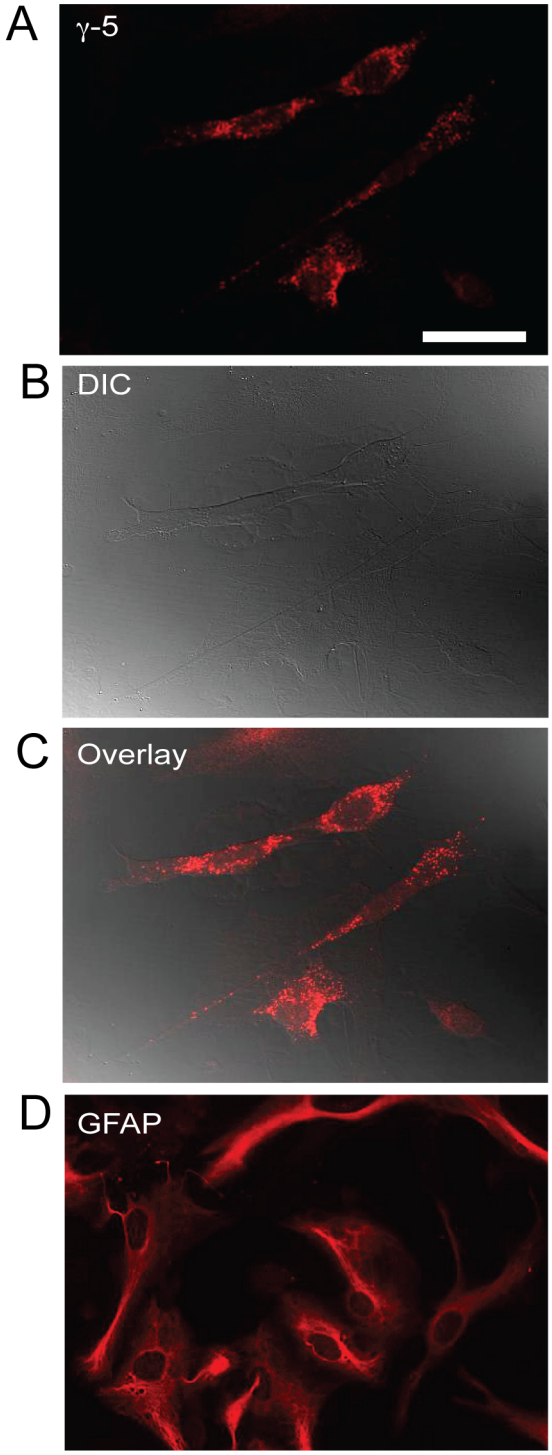


Figure 3.4. The TARP γ-5 is expressed in cultured fusiform glia from the cerebellum.

(A) Cultured cerebellum glial cells (days in vitro, (DIV7)) from P7 rats were labelled with rabbit anti-γ5. DIC images (middle panel) revealed fusiform glia labelled with γ-5. (**D**) To identify astrocytes in cerebellar cultures, cells were labelled with rabbit anti-GFAP.

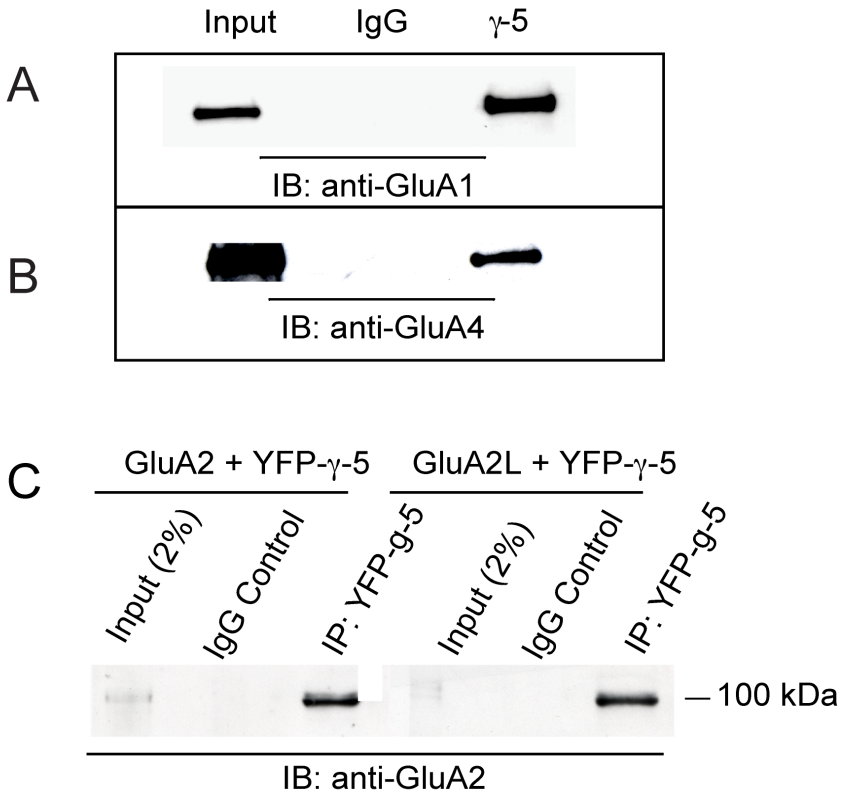


Figure 3.5. The TARP γ-5 interacts with native and recombinant AMPAR subunits.

(**A-B**) Western blots demonstrated the interaction of γ-5 with native GluA1 and GluA4 from rat cerebellum. P20 rat cerebellum was homogenised, initially a proportion of the cerebellum lysate was denatured using SDS (95 °C). This was used to determine the total amount of GluA1 and GluA2 expressed in the cerebellum (A, input). The remaining lysate was incubated with antibody against γ-5, which binds to the native γ-5 protein. To determine the specificity of the γ-5 association with AMPA receptors, the cerebellar lysate was also incubated with a non-immune antibody control (IgG). The membrane was probed with anti-GluA1 (**A**) and anti-GluA4 antibodies (**B**). (**C**) YFP-tagged γ-5 (YFP–γ-5) was transfected into tsA201 cells together with either GluA(Q)2 or GluA2L (Q). Both GluA(Q)2 and GluA2L (Q) co-immunoprecipitated with γ-5. YFP–γ-5 protein complexes were immunoprecipitated (IP) with anti-GFP and subjected to Western blotting with input samples and the IgG controls using the anti-GluA2 antibody. The nearest molecular weight marker is indicated.

3.3.6. γ-5 regulates AMPARs involved in neuronal-glial signalling

BGC AMPARs are activated by release of glutamate from climbing and parallel fibres in the cerebellum (Clark et al., 1997). Previous experiments, using double immunogold labelling, have shown that the scaffolding protein SAP97 (synapse-associated protein-97) is associated with GluA1 in the BGC plasma membrane directly facing parallel fibre terminals (Douyard et al., 2007). Is γ-5 important for neuronal-glial communication in BGCs? To determine whether γ-5 is present in a complex with SAP-97 I used co-immunoprecipitation assay from cerebellar tissue from the adult rats. These experiments revealed an interaction of SAP97 with both GluA1 and γ-5 in the cerebellum (**Figure 3.6A**). Fukaya et al., (2005) has shown that γ-5 mRNA expression in the cerebellum is restricted to BGCs, and as SAP-97 is also present in these cells at points of contact between CF-PFs and BGCs, our experiments suggest that BGC AMPARs at these contact sites are associated with γ-5.

Previous studies have revealed the presence γ-4 and γ-5 mRNA in BGCs (Fukaya et al., 2005). To determine whether γ-5 or γ-4 is the predominant TARP regulating BGC AMPAR responses, we next examined the published values for the decay of BGC quantal events (Matsui et al., 2005) and compared the published values with the deactivation kinetics of the recombinant receptors GluA4 and GluA1 expressed with and without TARPs γ-4 and γ-5. In accord with our co-immunoprecipitation data suggesting that SAP-97 and γ-5 formed complexes with AMPARs in BGC as the decay of quantal events matched well with the deactivation kinetics of receptors co-expressed with γ-5 (**Figure 3.6B**). Of note, the time constant for the deactivation of GluA1 or GluA4 receptors expressing γ-4

was more than five times that of the decay of BGC quantal events (**Figure 3.6C**). These results therefore support the view that suggesting that the properties of AMPARs involved in neuronal-glial signalling in BGCs are regulated by γ-5.

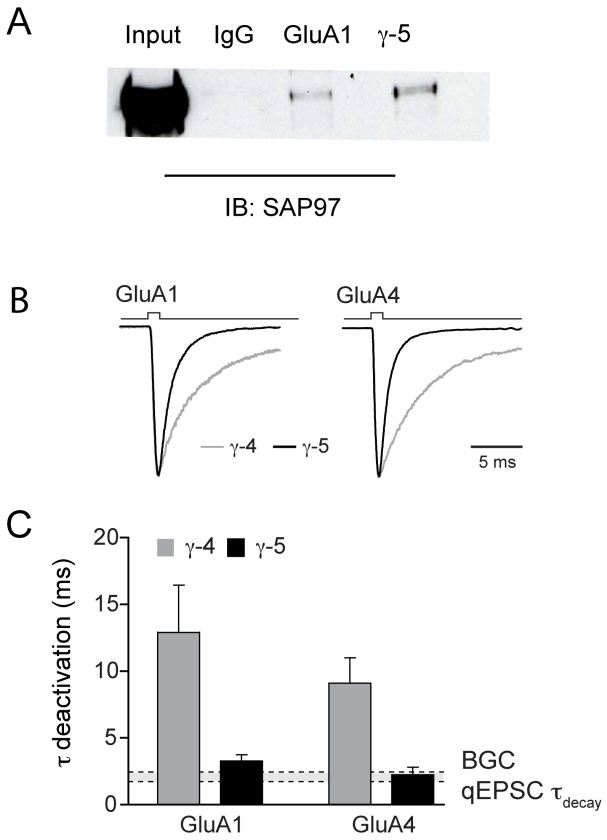


Figure 3.6. γ-5 is associated with transmitter activated AMPARs in BGCs.

(**A**) Western blot demonstrating co-immunoprecipitation of γ-5 with SAP97 in the rat cerebellum. Protein extracted from the adult rat cerebellum was incubated with either an antibody to γ-5 or an antibody to GluA1 or control IgG. (**B**) Representative averaged current responses after the application of glutamate (1 mM, 1 ms, -60 mV) to outside-out patches taken from tsA201 cells transfected with GluA1 or GluA4 AMPAR subunits co-expressed with γ-4 or γ-5. (**C**) Deactivation kinetics of GluA1 and GluA4 co-transfected with either γ-4 (grey) or γ-5 (black) in comparison to the quantal EPSC τ decay BGCs kinetics (dashed lines). The deactivation kinetics of GluA1 and GluA4 + γ-5 match closely with τ decay values for BGC qEPSC (dashed lines) (error bars are s.e.m). BGC AMPARs deactivation data taken from Matsui and Jahr (2006).

3.3.7. In olfactory bulb and spinal γ-5 is selectively expressed in astrocytes

TARPs are expressed in various neurons and glia throughout the central nervous system. TARPs γ-2, γ-3, γ-4, γ-7 γ-8 have been previously identified in neurons, such as cerebellar granule cells (γ-2) and hippocampal neurons (γ-8) (Chen et al., 2000; Tomita et al., 2003 Rouach et al., 2005). Furthermore, γ-4 has been identified in both neurons and astrocytes in the CNS (Tomita et al., 2003). However so far none of the TARPs (γ-2, γ-3, γ-4, γ-7 and γ-8) appear to regulate AMPARs in glia. γ-5 mRNA, which appears to be expressed in Bergmann glia in the cerebellum, is also expressed in the olfactory bulb of P7 rats. To obtain further insight into whether γ-5 is selectively expressed in glial cells, neuron-glia co-cultures were generated from the olfactory bulb and the spinal cord of E17 rats. Neurons were identified with an antibody against the neuronal microtubule-Associated Protein-2 (anti-MAP2), and glial cells were identified with an antibody against GFAP. The majority of co-cultures from the olfactory bulb and the spinal cord contained both MAP2-positive cells and GFAP-positive cells. In these cultures, the γ-5 was generally located to non-neuronal MAP2-negative cells, which had morphologies similar to that of GFAP positive astrocytes (**Figures 3.7 & 3.8**).

Previous studies have shown that in the cerebellum γ-5 does not co-immunoprecipitate with the postsynaptic density protein PSD-95, which is expressed at high levels in neurons (Kato et al., 2007). However as described above, in the cerebellum we find that γ-5 is in a complex with SAP-97, a protein highly enriched in glia. This observation appears to be consistent with the idea that γ-5 may be a glial specific-protein.

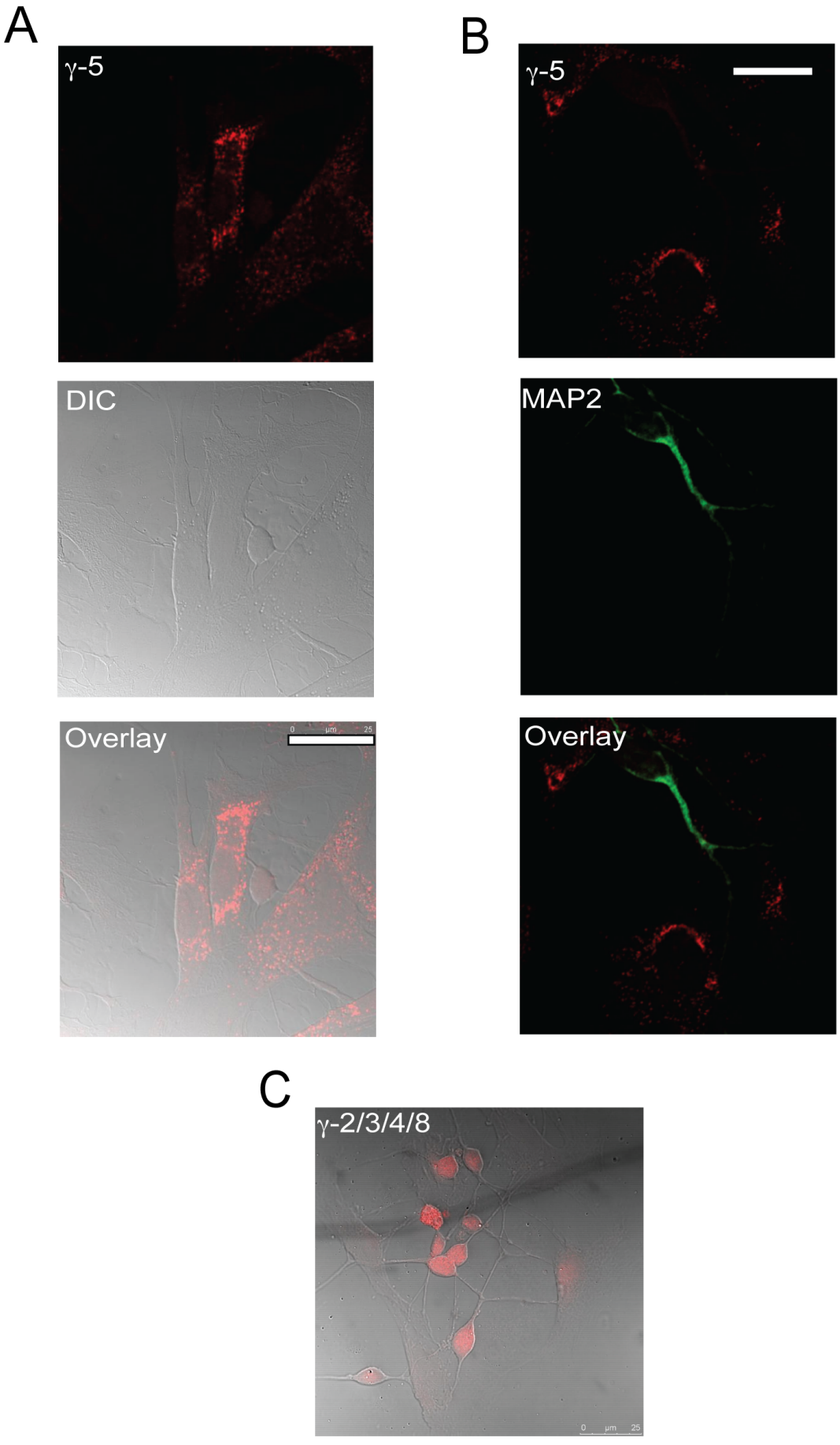

A
γ-5
DIC
Overlay
B
γ-5
MAP2
Overlay
C
γ-2/3/4/8

Figure 3.7. γ-5 is prominently expressed in non neuronal cells in the olfactory bulb .

(**A**) Mixed neuronal-glial cultures were obtained from P7 rat olfactory bulbs. Cells were labelled with rabbit anti-γ–5 (red) DIC image (middle) reveals high levels of expression in cells with astrocytic morphology, however cells with neuronal morphology are devoid of γ-5 expression. **(B)** γ-5 is not expressed in MAP2 positively labelled neuronal cells (green). In the overlay image, note the presence of γ-5 labelling in the large flat astrocytic cell (black arrow) and the absence of γ-5 expression in a neuronal MAP2-positive cell (white arrow) evident in the DIC image (lower panel). **(C)** Neuronal-astrocyte cultures were fixed and labelled with anti-γ-2,γ-3,γ-4,γ-8 (pan-TARP) (red). DIC images of neuronal-glial cultures labelled with pan-TARP antibody (red). Note the contrast in the expression patterns of the TARP γ-5 as seen in middle image of panel A, and the expression pattern of cell labelled with pan-TARP antibody **(image C)** (γ-2,γ-3,γ-4 and γ-8) as these proteins are expressed in cells with neuronal morphologies in contrast to γ-5. (Scale bar 25μM).

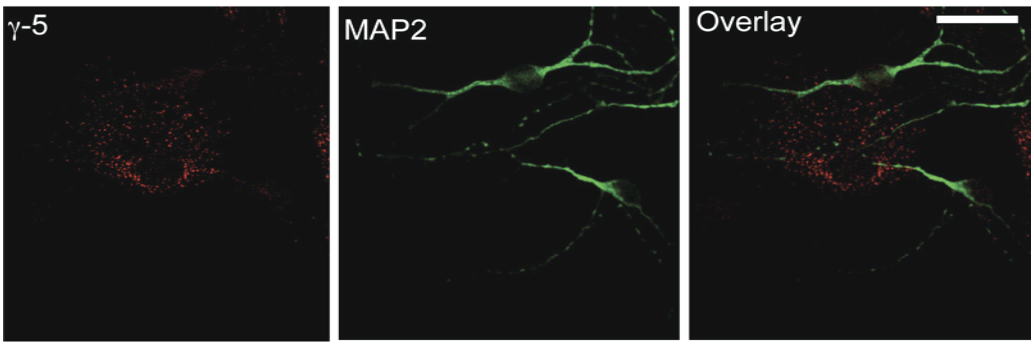


Figure 3.8. Glia in spinal cord express γ-5.

Spinal cord spiny neurons were grown on an astrocyte feeder layer. Neuronal-glial cultures were harvested from E15 rat embryos after six days *in vitro*. Cultures were labelled with rabbit anti-γ-5 **(A)** and anti-MAP2 **(B).** As is apparent from the overlay image, γ-5 labelling is predominant in astrocytes taken from e spinal cord. A very low level of expression was detected in spiny neurons (n=22 cells).

3.3.8. γ-5 decreased GluA2(Q) expression via the lysosomal pathway

The data described in section 3.3.3 suggested that the cell surface level of GluA2(Q) was decreased in the presence of γ-5. There are three possible explanations for this observation (1) GluA2(Q) was retained at the

endoplasmic reticulum or (2) GluA2(Q) was rapidly removed from the cell surface and sequestered in early endosomes, or (3) binding of γ-5 to GluA2(Q) initiates targeting of the receptor to the late endosomes or lysosomes for subsequent degradation. To determine which of the mechanisms could account for effects of γ-5 on GluA2(Q), we examined the localisation of γ-5 in cultured olfactory bulb astrocytes. Initially we investigated whether γ-5 was present in the endoplasmic reticulum (ER) by labelling cells with an anti-γ-5 antibody and an anti-protein disulphide isomerase marker (a protein expressed in the ER). In the majority of olfactory bulb glia, γ-5 and PDI did not appear to co-localise, suggesting that γ-5 was not retained in the ER. To examine whether γ-5 was localised in the early endosome, cells were labelled with the antibody for EEA1 a marker for early endosome, together with an anti-γ–5 antibody. There was no apparent co-localisation between γ-5 and the early endosome. **(Figure 3.9).** We next investigated whether this reduction was due to the degradation of GluA2(Q), we investigated whether γ-5 was significantly localised in the late endosomal pathway and hence, whether reduced surface expression of GluA2(Q) reflected degradation. The tSA201 cells were transfected with GFP-γ-5 and the late endosomal marker CD63. The co-localisation rate was calculated by comparing the percentage of overlay between the fluorescence of the GFP-γ-5 and CD63 using LEICA microscope co-localisation software. There was 63% co-localisation of γ-5 and the late endosome marker, strongly suggesting that γ-5 was targeting receptors in the lysosomes. To further address whether γ-5 was targeting GluA2(Q) to the lysosomes we performed cell surface biotinylation experiments in the presence of the lysosomal protease inhibitor leupeptin.

In the presence of this protease inhibitor, the cell surface levels of GluA2(Q) recovered to control levels (**Figure 3.10**).

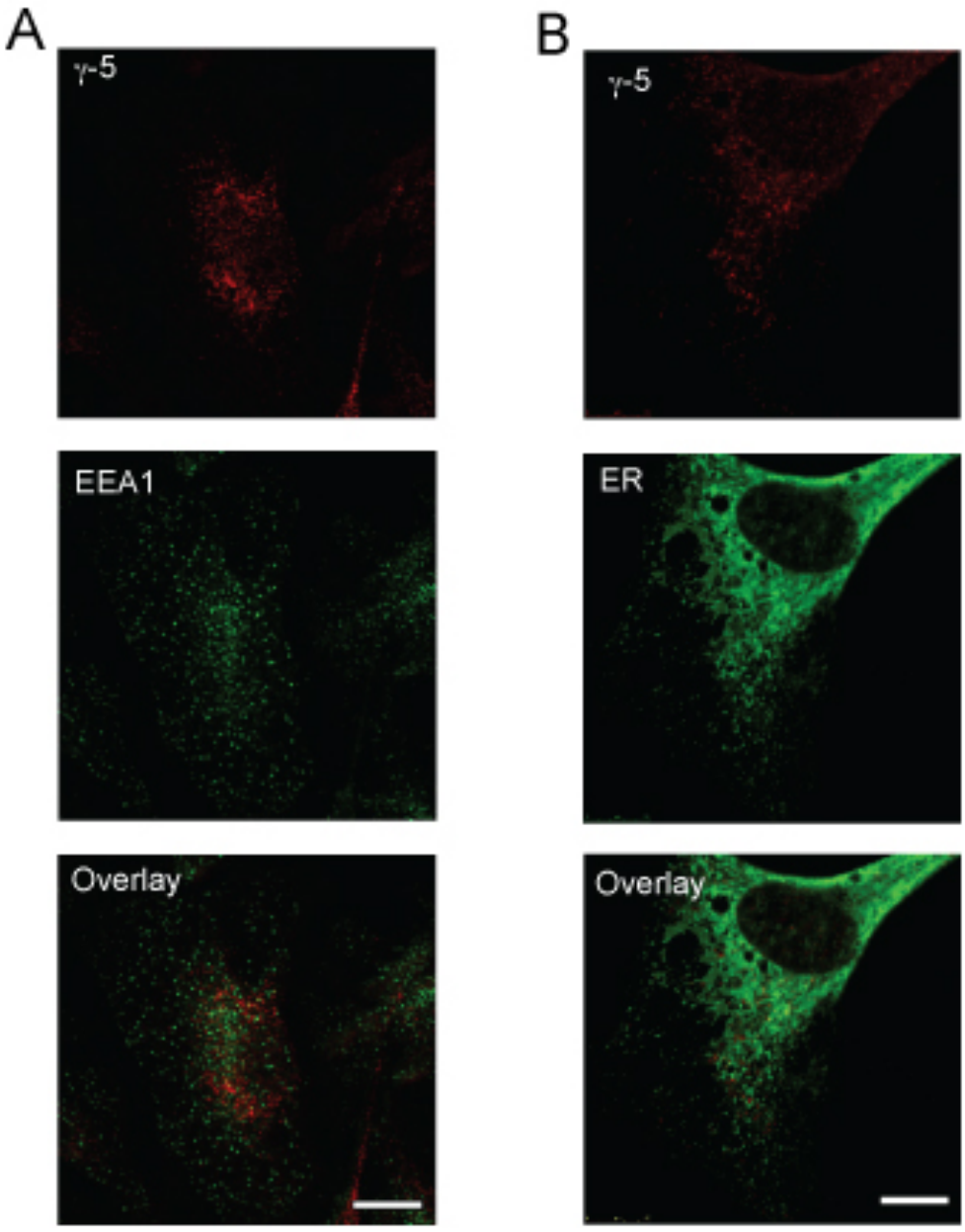


Figure 3.9. γ-5 does not co-localise with the early endosome or the endoplasmic reticulum in glia.

(A) Olfactory bulb glia (DIV12) were labelled with anti-γ-5 and the early endosome marker EEA1 (n = 28 cells). From the overlay image shown in image A, γ-5 does not appear to overlap significantly with the early endosome (P = 0.16). **(B)** Glial cells were labelled with a mouse anti-PDI (marker for the ER) and γ-5. The distribution of γ-5 did not overlay with the distribution of PDI (n=15) (Scale 25μm).

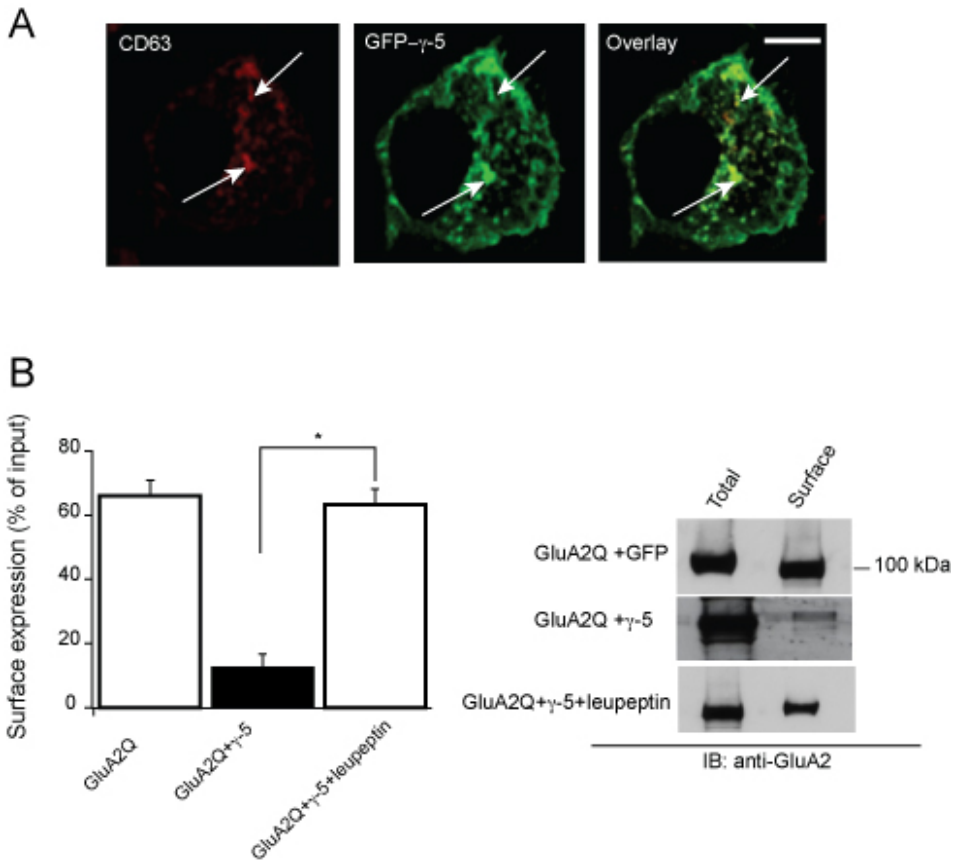


Figure 3.10. TARP γ-5 co-localises with the late endosome marker CD63.

(**A**) TSA201 cells were transfected with GFP tagged γ-5 (middle panel) and permeabilized with 0.5% Triton 24 hrs post-transfection. Cells were labelled with an anti-CD63 (right-hand panel) (n=30 cells). From the overlay image, co-localisation is apparent. The overlay of γ-5 and CD63 was 63%, which suggests that a proportion of the TARP resides in the late endosome pathway (co-localisation was analysed using Leica microscope co-localisation software). (**B**) Cells were transfected with GluA2(Q) and GFP, or with GluA2(Q) and γ-5. Cells transfected with GluA2 (Q) + γ-5 were incubated with 100 μM of the protease inhibitor leupeptin for 1 hour at 37°C to (n=3). Cell-surface biotinylation was performed to determine the percentage of surface GluA2(Q), when cells were co-transfected GluA2 (Q) with γ-5 and then treated with the protease inhibitor leupeptin. The application of leupeptin significantly prevented the loss of cell surface GluA2Q when expressed with γ-5. Bars in left hand panel of B are the mean values +/- S.E.M. $P<0.05$*

3.4. Discussion

3.4.1. γ-5 is a functional TARP

The experiments in this chapter suggest that γ-5 is behaving in some respects to TARP family members as it interacts with AMPARs and altering the trafficking and channel properties. However, unlike other TARPs, γ-5 exhibits selectivity for long-form AMPARs, the majority of which are calcium permeable. The results also show that γ-5 expression is largely restricted to non neuronal cells in a number of brain regions.

3.4.2. γ-5 is selective for long-form AMPA receptors

These experiments have established that γ-5 is a TARP, but that it displays a number of unique properties. In particular (1) increasing the channel conductance of the long-form subunits (GluA1 and GluA4) (2) decreasing the surface expression of AMPARs containing GluA2(Q) and (3) decreasing the channel open probability.

As most long-from AMPAR subunits in the CNS form CP-AMPARs, γ-5 modified predominately CP-AMPARs. The unique property of γ-5 could be attributed to differences in the amino acids, present in the C-terminus compared with the other identified TARPs. The TARPs γ-2, γ-3, γ-4 and γ-8 contain the PDZ-binding region -TTPV, which is responsible for interaction with PSD-95 and the clustering AMPARs at the synapse (Bats et al., 2007). However, the C-terminus of γ-5 expresses the amino acids –SSPC. This could interact with a different class of PDZ-binding proteins. Using a yeast two-hybrid screen against the C-terminus of the TARPs γ-2, γ-3, γ-4, γ-5, γ-7 and γ-8, Deng et al., (2006) demonstrated that, although γ-2, γ-3, γ-4, and γ-8 bind to PSD-95 and MAG-1 (membrane-associated guanylate kinase), neither γ-5 nor γ-7 can bind to these proteins, suggesting that they

may interact with distinct protein partners.

A further possible explanation for the unusual behaviour of γ-5 could be that it has an exceptionally short C-terminus and, consequently, it lacks of all but two of the ten-phosphorylation sites present in the C-terminus of other TARPs. Because these sites are involved in the regulation of AMPAR trafficking, it seems likely that these differences between TARPs could influence differential receptor regulation.

3.4.3. γ-5 has unique effects on cell surface AMPARs

The current experiments indicate an interesting feature of γ-5 in its ability to act as a suppressor of AMPARs cell surface expression. This reduction is selective for the calcium permeable short-form of GluA2 (GluA2(Q)). It would be interesting to perform a yeast-two hybrid screen against the N-terminal and C-terminal domains of γ-5 for proteins that are responsible for targeting the protein to the late endosomes.

3.4.4. γ-5 regulates AMPARs involved in neuronal-glial signalling

Within the cerebellum, only BGCs express γ-5 mRNA (Fukaya et al., 2005). These cells are known to express CP-AMPARs, which are activated during neuronal–glial signalling and are crucial for the maintenance of normal synaptic transmission from parallel and climbing fibres to Purkinje cells (Iino et al., 2001). Despite the importance of AMPARs in neuron–glia interplay, it was previously unknown whether TARPs influence the trafficking, anchoring or functional properties of AMPARs in glia. Current experiments indicate that CP-AMPARs in these BGCs have properties that match those shown by recombinant γ-5-associated AMPARs. The plasma membrane of BGCs that directly faces sites of ectopic glutamate release

from climbing and parallel fibres contains both SAP97 and GluA1 receptor subunits.

Previous studies have shown that γ-5 does not interact with PSD-95 (a protein highly enriched in neurons), suggesting that the γ-5 is absent in neuronal synapses (Kato et al., 2007). Because γ-5 in the cerebellum is restricted to BGCs, our finding that the γ-5 antibody pulled down SAP97 supports these previous observations. (Douyard et al., 2007) Together, our data suggest that γ-5 is associated with AMPARs in the BGC membrane directly opposed to presynaptic terminals and that it determines the properties of the CP-AMPARs underlying neuron–BGC signalling. Furthermore, the continuous activation of BGC CP-AMPARs by glutamate released from climbing and parallel fibres has been reported to be essential for the maintenance of connections to Purkinje cells (Iino et al., 2001).

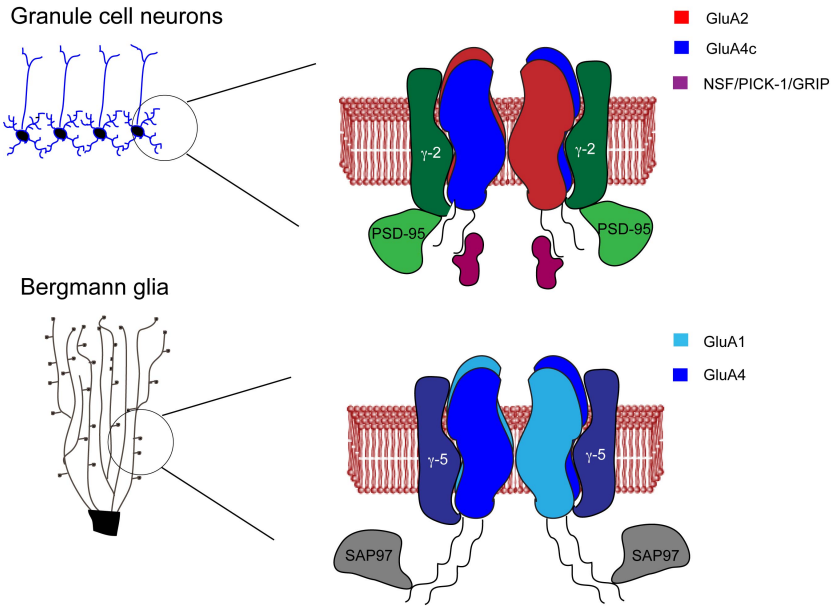


Figure 3.11. Hypothetical comparison of TARP-AMPAR interaction at neuronal synapses vs neuronal glial junctions.

γ–2 has been shown to interact with PSD-95 and with GluA2/GluA4 heteromers at neuronal synapses, in comparison to γ-5, which forms a complex with GluA1/GluA4 heteromers and with SAP-97 in Bergmann glia cells.

3.4.5. γ-5 is unique to glia in several brain regions

In this chapter we have shown that γ-5 present in the cerebellar, olfactory bulb, and spinal cord, where it could influence AMPAR-mediated calcium entry. The inability of γ-5 to 'rescue' AMPAR responses in TARP-lacking cerebellar granule cells from stargazer mice is entirely consistent with our findings. Granule cells express predominantly short-form AMPAR subunits (GluA2 and GluA4c). We found only a small increase in channel conductance when GluA2/GluA4 heteromers were co-expressed with γ-5. This is consistent with the fact that γ-5 did not enhance AMPAR surface expression, in cerebellar granule cells, suggests that γ-5 would be unlikely to alter the macroscopic current of short-form containing AMPARs.

A recent study published by (Kato et al., 2008) also shows that γ-5 is a TARP with subunit selectivity. However, the conclusions produced from theis study differs notably from the present work in this chapter as it argues that; (i) only GluA2-containing AMPARs are regulated by γ-5, (ii) this specificity depends on editing at the Q/R site of GluA2, and finally (iii) γ-5 does not control the receptor trafficking or surface expression of AMPARs. Although we found, in one case (homomeric GluA2 AMPARs), that γ-5 had apparent editing-dependent effects, these recent findings contrast with our main conclusion that γ-5 interacts with all AMPAR subunits and, to varying extents, modifies their properties. In the present study, the predominant functional effect of γ-5 was on long-form AMPARs, which, with the exception of the splice variant GluA2L, are calcium permeable. This selectivity of γ-5 of that I have described was not dependent on the Q/R editing state. The presence and function of γ-5 in BGCs, a cell type that expresses only CP-AMPARs, seems inconsistent with the view that this TARP affects only GluA2-containing AMPARs.

Chapter 4. ATP and glutamate mediate TARP dependent AMPAR bidirectional plasticity in oligodendrocyte lineage cells

4.1. Summary

Oligodendrocyte precursor cells (OPCs) are a major glial cell type in the CNS. These cells express a mixture of calcium permeable and calcium impermeable AMPARs, as well as phospholipase C linked G-protein coupled group I mGluRs and purinergic P2Y receptors (Itoh et al., 2002;Luyt et al, 2004). Initially we examined whether activation of mGluRs in optic nerve OPCs can mediate AMPAR plasticity, similar to that previously described in the cerebellar stellate cells synapses where mGluR1 activation drives the loss of calcium permeable AMPARs. However in striking contrast to mGluR response in stellate-cell interneurons the activation group I mGluR in OPCs promoted an increase the proportion of calcium permeable AMPARs.

This plasticity in OPCs was independent of CamKII and utilized kinases similar to that used to control mGluR dependent cerebellar LTD. We also investigated whether mGluR mediated AMPAR plasticity could be induced in mature pre-myelinating OPCs, and found a decrease in mGluR mediated AMPA plasticity in these cells.

Neurons and astrocytes in the white matter have been shown to release ATP along with glutamate onto OPCs stimulating an increase in intracellular calcium in these cells (Hamilton et al., 2010; Porter and McCarthy, 1996; Wang et al., 2000; Zhang et al., 2003). Our experiments suggest that activation of purinergic receptors can also regulate the relative proportions of calcium permeable AMPAR. Finally our experiments show

that the transmembrane AMPA regulatory protein stargazin (γ-2) regulates AMPAR properties in OPCs, and that the PDZ binding domain of γ-2 is critical for mGluR-mediated AMPA plasticity in these cells.

4.2. Introduction

Oligodendrocytes are the major myelinating cells in the mammalian CNS. These cells derive from oligodendrocyte precursor cells (OPCs) that arise in spatially restricted regions of the ventricular zone (Noble, 2000; Rogister et al., 1999).

Native OPCs derived from the optic nerve express a variety of receptors including calcium permeable-AMPARs, also expressed in a primary OPC cell line (CG4), which can also fully differentiate into oligodendrocytes (Pende et al., 1994). Glutamate has been shown to potentiate the expression of immediate early genes, and influence OPC proliferation and migration (Gudz et al., 2006; Pende et al., 1994) Furthermore, activation of CP-AMPARs in these cells has been linked to an increased risk of their apoptosis after acute periods of hypoxia (Alberdi et al., 2002).

Bergles et al., (2000) provided evidence that CP-AMPARs expressed on OPCs are activated by glutamate released from CA1 hippocampal neurons. This release of glutamate from neurons onto OPCs can induce AMPAR plasticity in the hippocampus producing an increased expression of CP-AMPARs (Ge et al*.,* 2006). Although the mechanisms underlying this change are unknown it is interesting that OPCs are also know to express mGluRs receptors providing a potential mechanism for controlling AMPAR plasticity in OPCs. The experiments described in this chapter demonstrate that activation of mGluRs and P2Y receptors can give rise to a form of bidirectional plasticity in OPCs, our experiments also suggest that the

TARP γ-2 is expressed in OPCs, and is involved in the mGluR mediated AMPAR plasticity in these cells.

4.3. Results

4.3.1 mGluR activation decreases AMPAR channel rectification in CG4-OPCs

To examine whether mGluR activation might alter the proportion of GluA2-containing CP-AMPARs in OPCs, we examined glutamate-evoked whole-cell current-voltage (I-V) relationships in CG4 OPCs (an OPC cell-line). We assessed the presence of GluA2-lacking CP-AMPARs from the voltage-dependent block produced by added intracellular spermine (100 μM). In these experiments, the agonist solution contained 100 μM glutamate plus 50 μM cyclothiazide to prevent AMPAR desensitization. We first tested whether CG4 cells generate rectifying responses that could be blocked by the AMPAR antagonist GYKI 52466 dihydrochloride (50 μM) (**Figure 4.1**). In control untreated cells, the I-V relationships (–100 to +60 mV) showed modest rectification, with a mean rectification index (RI, **see Methods)** of 0.68 ± 0.04, n = 6) indicating a mixture of calcium permeable and calcium permeable AMPARs present at the cell surface of in CG4 OPCs (**Fig. 4.2A**). Having shown the presence of CP-AMPARs in these cells we next examined the effects of group 1 mGluR (mGluR1/5) selective agonist (*S*)-3,5-dihydroxy-phenylglycine (DHPG, 100 μM; 30 minutes at 37°C). Following mGluR activation the I-V relationships became more rectifying (**Figure 4.2B**), with RI reduced to 0.33 ± 0.05, n = 10; P = 0.0006***). mGluR induced decrease in AMPAR rectification index was reduced with mGluR5 antagonists ACDPP applied together with the group I/II mGluR antagonist MCPG (RI = 0.055 ± 0.04 P= 0.08 compared to control RI). This

change in rectification is consistent with an increase in the proportion of CP-AMPARs following DHPG treatment (**Figure 4.2D**). At negative potentials (–100 mV), where CP-AMPARs are relatively unaffected by polyamine block (Bowie and Mayer, 1995; Soto et al., 2007), DHPG application increased the current density from 64.1 ± 15 pA/pF to 158.1 ± 31 pA/pF (n = 10 and n = 10; P = 0.00016). Such an increase could reflect a simple increase in receptor number, but would also be consistent with the higher single-channel conductance of CP-AMPARs compared with calcium impermeable-AMPARs (Feldmeyer et al., 1999; Mameli et al., 2007). The current density increase produced by DHPG could be prevented by application of a mixture of mGluR blockers ACDPP (10 μM) and MCPG (500 μM) (**Figure 4.2D**). The current density in the presence of DHPG+MCPG+ACDPP was 83.3 ± 16.22 pA/pF were not significantly different from control values (n = 6; P = 0.09).

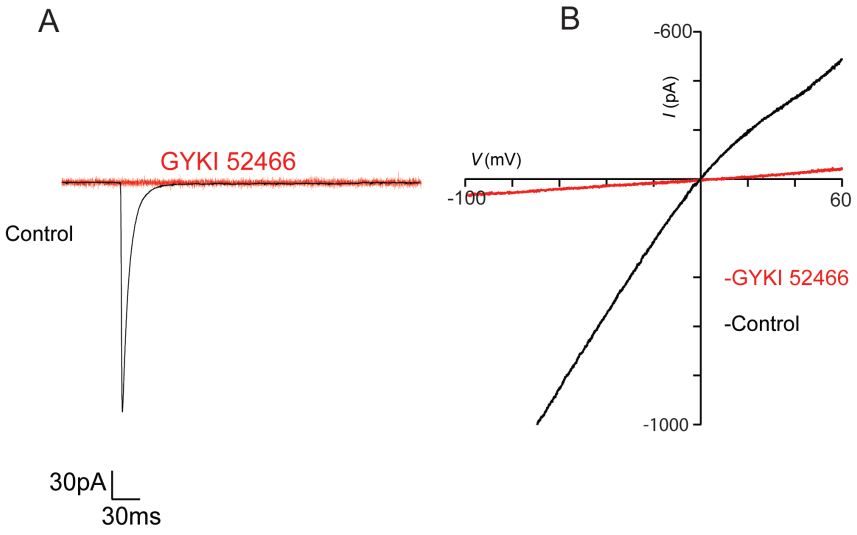


Figure 4.1. Glutamate activates AMPARs in CG4 OPCs.

(A) Out-side out patches pulled from CG4 OPCs cells were exposed to 100μM glutamate for 100ms, activating AMPARs, which was blocked with the application of 50μM GYKI 52466. **(B)** Whole-cell AMPAR current ramps in response to glutamate were blocked by GYKI 5246.

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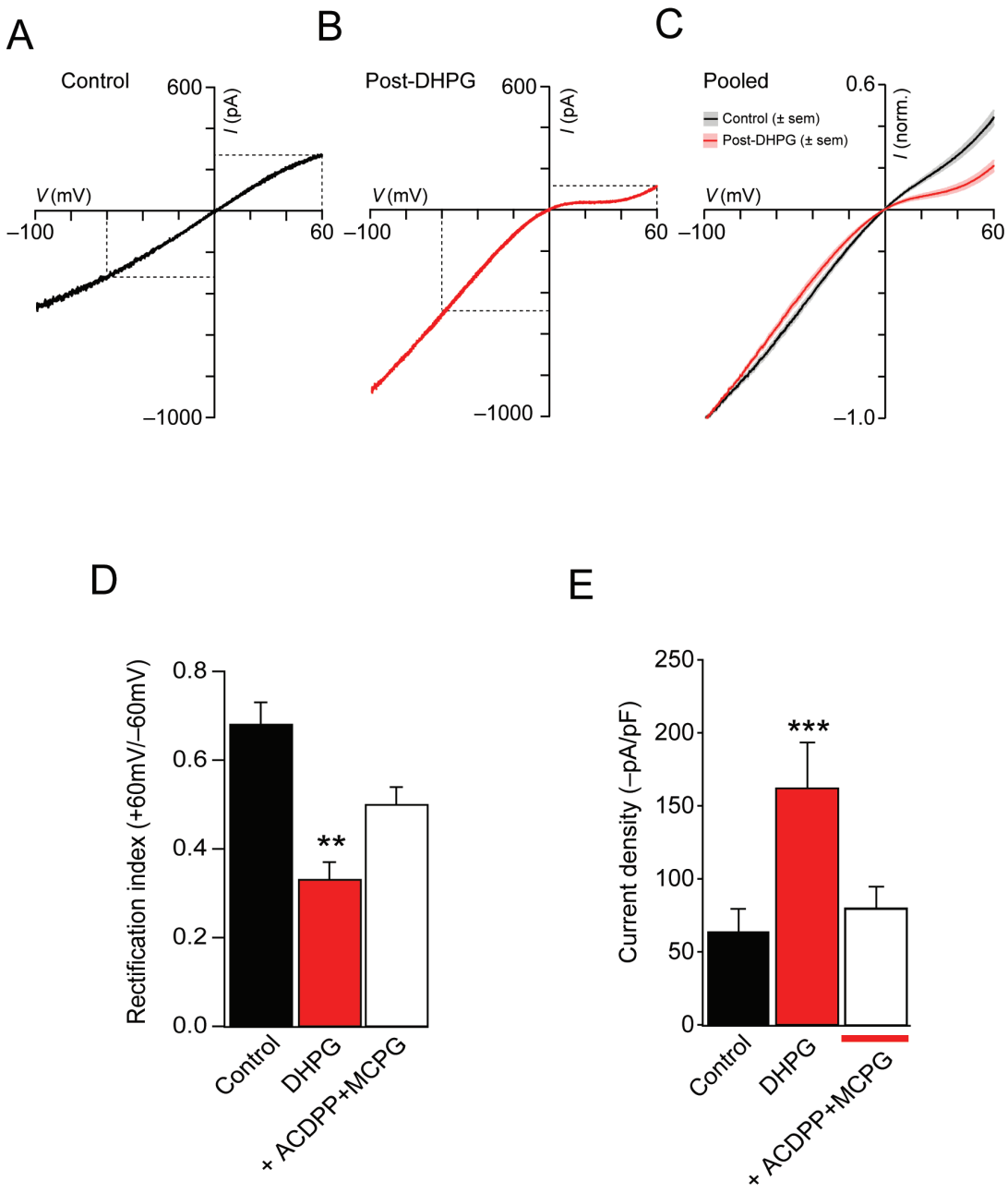


Figure 4.2. Group I mGluR agonist DHPG increases the proportion of calcium permeable AMPARs in CG4 OPCs.

(A-B) Typical example of whole-cell I-V AMPAR responses (+60mV to -100mV) from control cells and cells treated with the 100μM of the mGluR agonist DHPG, **(C-D)** Averaged whole-cell ramp I-V responses to bath application of 100μM glutamate from control cells (n = 6), and DHPG treated cells (n = 10). DHPG treated cells had an inwardly rectifying response and a significantly reduced Rectification Index (current amplitude at +60mV/-60mV); (Control RI= 0.68 ± 0.04, DHPG treated cells RI= 0.33 ± 0.05; n = 6 and n = 10; P = 0.0006**). (**D**) The

mGluR antagonist ACDPP (10μM) together with MCPG (500μM) blocked the DHPG induced reduction in RI (ACDPP+MCPG+DHPG, RI= 0.55 ± 0.04;n = 6). **(E)** DHPG increases the current density of AMPAR responses compared with control levels (control 64.1 ± 15 pA/pF n = 10; DHPG 158.1 ± 31 pA/pF n = 10 and *P* =0.00016***). The effects of DHPG on AMPAR current density was blocked by application of the mGluR antagonists ACDPP and MCPG (83.38 ± 16.22 n = 6) *** Denotes significant difference from control, D. Mean data and error bars represent S.E.M.

4.3.2. mGluR activation increases AMPAR channel conductance in CG4 cells

As discussed above, if DHPG treatment does indeed increase the proportion of CP-AMPARs in CG4 OPCs, one would expect an increase in the AMPAR single-channel conductance. To test this, we recorded currents from outside-out membrane patches (–60 mV) in response to rapid application of 10 mM glutamate (100 ms) and used non-stationary fluctuation analysis (NSFA; **see Methods**) to determine the weighted mean single-channel conductance and the peak open probability ($P_{o,\ peak}$). DHPG treatment produced a significant increase in single-channel conductance (Control 29 ± 2.3pS to DHPG 46 ± 4pS;n = 14 control and n = 11 DHPG; P = 0.0008) that was blocked by treatment with mGluR antagonists MCPG and ACDPP (35.2 ± 4.4 pS; n = 5, *P* = 0.29 compared to control) (**Figure 4.3D**). $P_{o,\ peak}$ and the time course of desensitization were not significantly affected by mGluR activation (**Figure 4.3E-F**).

Together the change in RI and single channel conductance suggest that activation of group I mGluRs in CG4 OPCs is producing an increase in the proportion of CP-AMPARs. This DHPG-induced change in AMPAR properties differs strikingly from that recently described in dopaminergic cells of the ventral tegmental area (that express CP-AMPARs following

cocaine treatment) (Mameli et al., 2007) and in molecular layer interneurons of the cerebellar cortex (Kelly et al., 2009). In these cases mGluR activation causes a *decrease* in the proportion of CP-AMPARs. Although these neuronal changes may differ in their details, they are both distinct from mGluR-induced long-term depression (LTD) seen in hippocampal CA1 cells and LTD at parallel fiber–Purkinje cell synapses (Moult et al., 2006; Xiao et al., 2001; Zakharenko et al., 2002) and instead reflect changes in protein synthesis and/or intramembrane movement of receptors, consequent upon elevation of intracellular calcium ($[Ca^{2+}]_i$).

4.3.3. DHPG-induced AMPAR changes require Ca^{2+} elevation

We next investigated potential mechanisms that might contribute to the mGluR-induced AMPAR changes in CG4 OPCs, by examining the effects of various treatments on the ability of DHPG to alter either RI or channel conductance. In OPCs, DHPG is known to increase intracellular $[Ca^{2+}]_I$, that is prevented by pretreatment with the selective mGluR antagonists MCPG and ACDPP, and results from inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from intracellular stores (Luyt et al., 2003). To determine whether such Ca^{2+} elevation might trigger the AMPAR changes we observed, we incubated CG4 OPCs with the membrane-permeable Ca^{2+} chelator BAPTA-AM (20μM) with DHPG. Following such treatment, the effect of DHPG on AMPAR channel single-channel conductance was suppressed (34.9 ± 5.0 pS; n = 9; P = 0.36 compared to control) **(Figure 4.3D).**

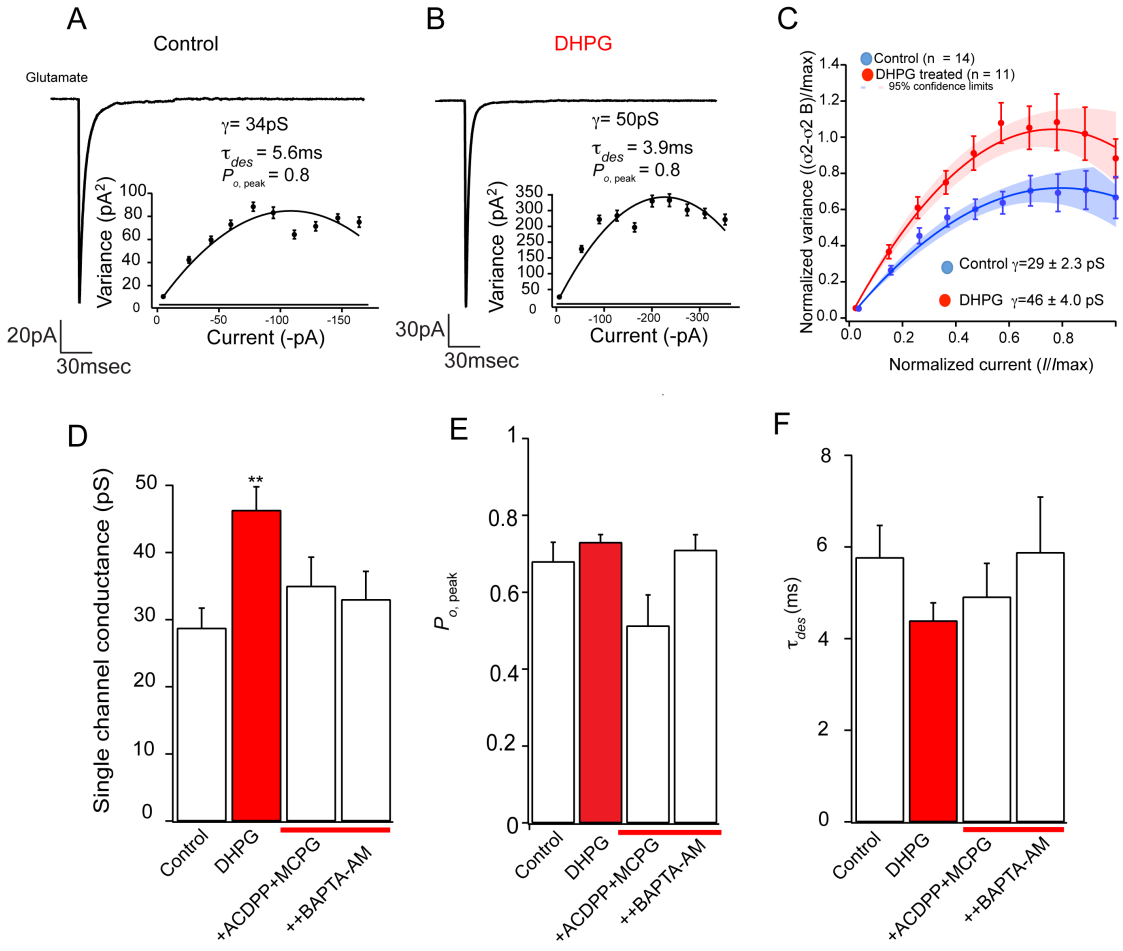


Figure 4.3. mGluR activation increases single-channel conductance of AMPARs in CG4 cells.

(A-B) Average responses to fast application of 10mM glutamate (100ms, -60mV) onto outside-out patches from control and cell treated with DHPG. Control currents are the mean of 80 traces; DHPG treated current is the mean of 110 traces. Insets show plots of noise variance vs. inward current. (**C**) Pooled current variances parabolas from control and DHPG treated cells. **(D)** The estimated mean single-channel conductance increased from control 29 ± 2.3 to DHPG treated 46 ± 4pS (P = 0.0008**). The mGluR mediated increase in single-channel conductance was blocked by the addition of the antagonists ACDPP and MCPG (35.2 ± 4.4 pS; *n* = 5, P = 0.29 compared with control). BAPTA-AM also blocked the rise in single channel conductance (single channel conductance = 34.9 ± 5.0pS P = 0.3 compared to control). **(E-F)** mGluR activation did not significantly increase AMPAR channel $P_{o,peak}$ nor alter AMPAR desensitization kinetics (control $P_{o,peak}$ = 0.68 ± 0.05; following DHPG treated = 0.73 ± 0.04; control τ_{des} = 5.77ms; following DHPG treatment τ_{des} = 4.39ms).

4.3.4. mGluR induced AMPAR plasticity is dependent on PICK-1, PI3K and protein synthesis

mGluRs mediated AMPAR plasticity in neurons has been linked to the activation of several key intracellular pathways that are regulated by phosphatidylinositol 3-kinases (PI3-K), c-Jun N-terminal kinases-3 (JNK-3) and protein interacting with C-kinase-1 (PICK-1) (Hou and Klann, 2004; Jo et al., 2008; Li et al., 2007). We therefore wanted to examine whether in mGluR-induced AMPA plasticity in CG4 cells was also dependent on these intracellular pathways.

In this study we first examined the PI3K-Akt-mTOR pathway, which has been shown to trigger an increase in local protein synthesis of GluA2, in response to mGluR1 activation in VTA neurons and stellate cell interneurons. This increase in protein synthesis was subsequently blocked by protein synthesis inhibitor cyclohexamide (C-hex) (Mameli et al, 2007; Kelly et al., 2009). To examine whether the mGluR induced decrease in AMPAR rectification in CG4-OPCs is dependent on the activation of PI3K and local protein synthesis, cells were treated with either 25μM C-hex or with the PI3K inhibitor wortmannin (co-applied with 100μM DHPG) for 30 minutes (**Figure 4.4B)**. C-hex and wortmannin significantly reduced the effect of DHPG activation on AMPAR rectification, providing evidence that newly formed CP-AMPARs are required for mGluR-induced AMPAR plasticity in CG4 cells (Control RI = 0.60 ± 0.055, C-hex + DHPG RI = 0.66 ± 0.08, wortmannin+DHPG= RI = 0.5 ± 0.039).

Next we examined whether the protein PICK-1 was involved in mGluR plasticity in CG4 cells. PICK-1 has been previously linked with mGluR induced internalisation of AMPARs in the perirhinal cortex (Jo et al., 2008). To test whether mGluR activation in CG4s triggers PICK-1 to internalize GluA2, the selective cell-permeable PICK-1-GluA2 blocking peptide TAT-

pep2-EVKI (25μM) was used. Cells were treated with a selective cell-permeable PICK-1 inhibitor peptide (25μM) together with 100μM DHPG. PICK-1 inhibitor peptide significantly inhibited the effect of DHPG, suggesting that activation of mGluRs is trigger internalisation of GluA2 via activation of PICK-1. (PICK-1 + DHPG RI = 0.66 ± 0.04; n = 7, P = 0.6 compared with control) (**Figure 4.4A**).

NMDA-induced LTD in CA1 hippocampal neurons has been shown to be dependent on CamKII (Leonard et al., 1999, Lee et al*.,* 2009). Currently no studies have reported whether mGluR mediated AMPAR plasticity could be dependent of activation of CamKII. To address this CG4 cells were treated with KN-62, a cell permeable blocker of CamKII. KN-62 failed to block the mGluR-induced reduction in AMPAR rectification in these cells (KN-62 RI = 0.4 ± 0.05; n=7, P = 0.001*), demonstrating that in CG4 the activation of mGluR is independent on CamKII (**Figure 4.4C**).

Finally I examined the role of the kinase JNK (JNK1-3), which are activated by glutamate binding to mGluRs. In recent studies Ahn et al (2009) demonstrated that the activation of group I mGluRs in the dorsal striatum leads to an increased phosphorylation of GluA2, which was be blocked with the application of cell permeable JNK antagonist (SP600125). (Thomas et al., 2008) has also shown that in neurons JNK-3 is responsible for the recycling of a long variance of GluA2. To examine whether JNK activation in CG4 cells controls AMPAR plasticity, cells were treated with the JNK antagonist SP600125 (100μM) together with DHPG (100μM). The blockade of JNK significantly reduced mGluR mediated AMPAR plasticity in CG4 (SP600125 RI= 0.6 ± 0.08 compared with control; n=8, P = 0.71) **(Figure 4.4A).** These results suggest that mGluR induced AMPAR plasticity in required activation of PI3K, PICK-1 and JNK-3 in CG4 cells.

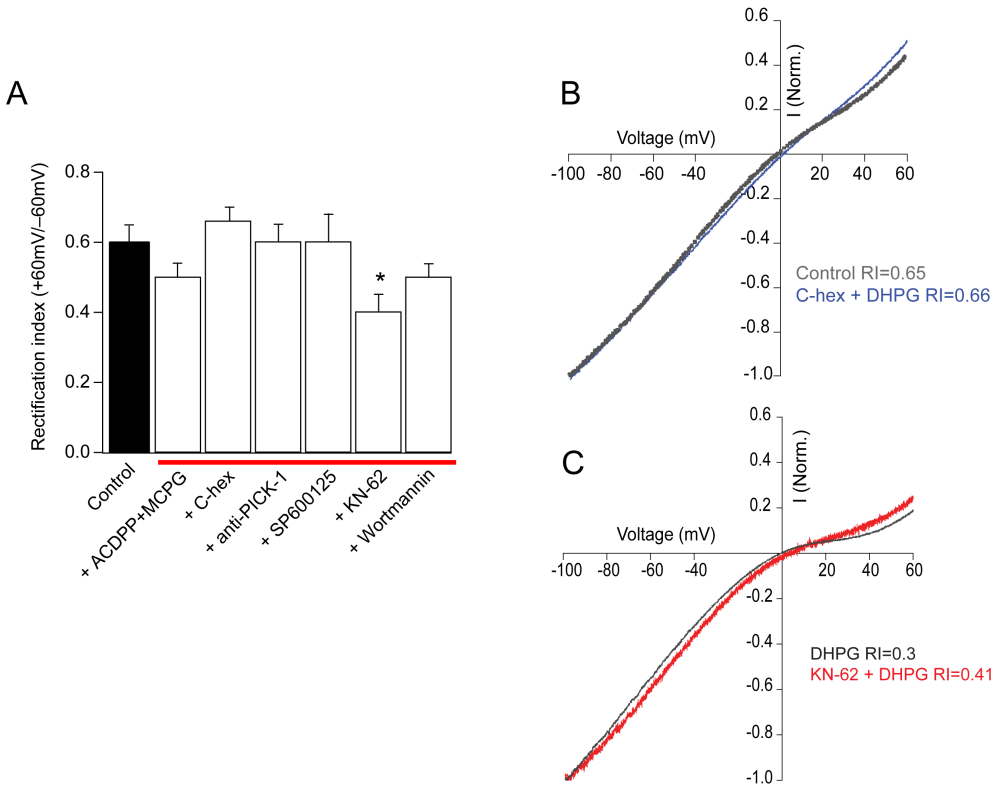


Figure 4.4. mGluR induced AMPA plasticity is dependent on intracellular kinases and protein synthesis.

Protein synthesis inhibitor cyclohexamide (C-hex) and PI3K inhibitor wortmannin (n = 7) or anti-PICK-1 peptide (20μM) (n = 7) suppressed mGluR induced change in rectification (Control RI = 0.60 ± 0.055, RI = C-hex + DHPG RI = 0.66 ± 0.08 P = wortmannin+DHPG = RI = 0.5 ± 0.039). JNK inhibitor SP600125 (n = 8) also perturbed the effect of DHPG on AMPA receptor rectification (SP600125, RI = 0.6 ± 0.08 compared to control P = 0.71) (Anova Kruskal-Wallis rank sum test). However, the CamKII inhibitor KN-62 failed to block DHPG induced decrease in rectification: RI = 0.4 ± 0.05 (P = 0.001*).

4.3.5. mGluR induced AMPAR plasticity in optic nerve OPCs is developmentally regulated

In the previous sections we focused on mGluR mediated AMPAR plasticity in CG4 cells. It was of importance to determine whether mGluR mediated AMPA plasticity was present in native OPCs. In this study we used purified OPCs from optic nerve, as this region contains a high density of OPCs whilst being devoid of neuronal cell bodies. Initially we examined whether mGluR-mediated AMPAR plasticity in native OPCs using DHPG. The AMPAR rectification was obtained using whole-cell I/V plots in response to glutamate. Consistent with CG4 cells, the activation of mGluRs in immature OPCs induced a reduction in AMPARs rectification index (Control RI = 0.7 ± 0.04 DHPG RI = 0.47 ± 0.02; Control n = 13, DHPG n = 13; P = 0.006*) (**Figure 4.5B**).

During development OPCs undergo differentiation into myelinating oligodendrocytes. During this differentiation, there are significant alterations in the receptor expression levels followed by an increase in the number of process formed (Dugas et al., 2010). It is possible to regulate the development of OPCs *in vitro* with the addition of the growth factors: platelet-derived (PDGF) and basic fibroblast (bFGF) that maintain OPCs in an immature phase. However the removal of mitogens and the addition of thyroxine to the OPC growth media triggers differentiation of the cells into pre-myelinating OPCs followed by reduced expression of GluA3 mRNA (Itoh et al., 2002; Temple and Raff, 1986). Similarly there are reductions in the expression of mGluR5 in mature pre-myelinating CG4 cells (Luyt et al., 2006). To assess whether mGluR plasticity is also present in premyelinating cells, we examined the AMPAR rectification in response to

glutamate after treating cells with DHPG. We also examined whether there were alterations of AMPAR channel properties during OPC differentiation into pre-myelinating cells. The AMPAR rectification index of pre-myelinating OPCs was larger compared to immature OPCs, suggesting a reduction of CP-AMPARs during differentiation of OPCs (immature OPC RI = 0.7 ± 0.04 n = 19, Pre-myelinating OPCs RI= 0.94 ± 0.02; n = 13; P = 0.04* Mann-Whitney U test) (**Figure 4.5E**-**G).** This was accompanied by decrease in the AMPARs single-channel conductance in pre-myelinating OPCs (Control OPCs 35 ± 2.8; n = 10) pre-myelinating OPCs (21.61 ± 1.3 n = 5; P = 0.01** Man-Whitney U test) **(Figure 4.5H).** These results are consistent with a reduction in CP-AMPARs in pre-myelinating OPCs. However this effect could also be attributed to a loss in the expression levels of mGluR5.

To examine whether there were alterations in mGluR mediated plasticity during OPCs differentiation pre-myelinating cells were exposed to DHPG (100μM) and the AMPAR mediated current rectification was measured using whole cell I/V ramps in the presence of 100μM glutamate + cyclothiazide. Interestingly, the mGluR-mediated plasticity of AMPARs was absent in pre-myelinating OPCs (Control RI= 0.94 ± 0.02 DHPG RI= 1 ± 0.06; P = 0.12 (**Figure 4.5E**). These results confirm that the expression levels of CP-AMPARs are downregulated as previously suggested from biochemical assays (Itoh et al., 2002). Importantly, we show that mGluR mediated AMPARs plasticity is developmentally regulated as it is absent in pre-myelinating cells.

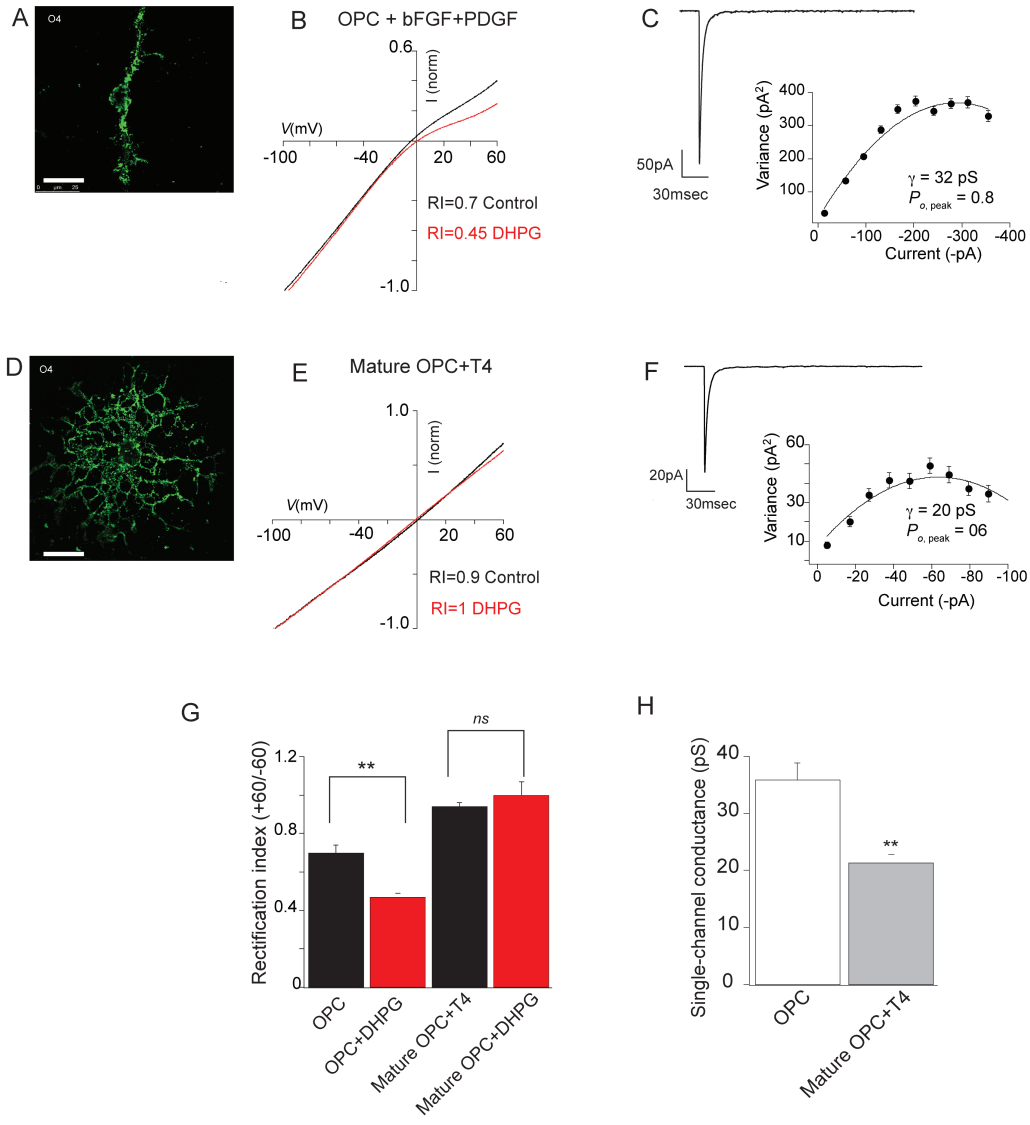


Figure 4.5. mGluR induced change in CP-AMPARs is developmentally regulated in optic nerve OPCs.

(A) Typical example of an optic nerve OPC (treated with PDGF and bFGF for 6 days *in vitro*) identified with the marker O4. **(B)** Averaged ramp I-V plots (+60mV to -100mV) obtained from OPCs exposed to bath application of 100μM glutamate.+ cyclothiazide. Control OPCs (black I-V) (n = 13), were compared with DHPG (100μM, 30 mins) treated cells (red I-V) (n = 13). DHPG treatment significantly reduced RI (current amplitude at +60mV vs -60mV) from a control value of RI = 0.7

± 0.04, to RI = 0.47 ± 0.02 (P = 0.006**). **(C)** Immature OPC average AMPAR responses from outside-out patch exposed to fast application of 10mM glutamate (100ms, -60mV). Insets show plots of noise variance *vs* inward current. **(D)** Example of pre-myelinating OPC (identified with the marker O4) obtained by starving immature OPCs of growth factors and addition of thyroxine for 48hours. **(E-G)** Averaged ramp I-V plots (+60mV to -100mV) obtained from pre-myelinating OPCs exposed to bath application of 100μM glutamate+ 50μM cyclothiazide. Control pre-myelinating OPCs (black I-V) (n = 6), were compared with DHPG (100μM, 30 mins) treated cells (red I-V) (n = 6). mGluR activation did not alter the AMPAR RI (current amplitude at +60mV/ 60mV) from a control value of RI = 0.94 ± 0.02, to DHPG treated RI = 1 ± 0.06 (P = 0.12; unpaired t-test) **(F)** Average responses from outside-out patch exposed to fast application of 10μM glutamate (100ms, -60mV) in pre-myelinating OPCs. Inset shows plot of noise variance *vs* inward current **(H)** The single-channel conductance of AMPARs in OPCs decreased during development from 35pS ± 2.8 (n = 10) in OPCs, to 21.61 ± 1.349 (n = 5) in adult cells (P = 0.01**). Statistics used were unpaired t-test and Anova.

4.3.6. Activation of P2Y receptors increases insertion of GluA2 containing AMPARs in the surface of optic nerve OPCs

The previous experiments have focused on mGluR meditated AMPAR plasticity in optic nerve OPCs. mGluRs in these cells are potentially activated by the release of glutamate from axons. However ATP is also thought to be released from optic nerve axons (Hamilton et al., 2010). To investigate whether application of ATP could also modify AMPAR properties in OPCs. Cells were exposed to 1mM ATP for 10minutes. Initially, we measured the effects of ATP on whole-cell AMPAR rectification index (+60/-60). In contrast with mGluR activation, ATP produced a

significant increase in rectification index compared with control cells 0.55 ± 0.07 to ATP treated RI= 0.911 ± 0.1 (n = 6 and n = 8) (P = 0.005**). This change in is indicative of an increase in the proportion of calcium impermeable at the surface of OPCs following treatment with ATP, and contrasts with the increase in CP-AMPARs that follows DHPG treatment.

The effects of ATP on AMPAR plasticity could inhibited with the P2 receptor antagonist PPADS (10μM) (PPADS+ATP RI= 0.522 ± 0.04 n = 5 P = 0.78 compared with control). To investigate whether ATP mediated AMPA plasticity was mediated through the G-protein coupled receptor (GPCR) P2Y receptor, cells were treated with ATP and the cell permeable PLC inhibitor RHC-80267 (20μM). RHC-80267 blocked the effect of ATP on AMPA plasticity (RHC-80267 +ATP RI = 0.63 ± 0.03; n = 6; P = 0.7). These results strongly suggest that activation of P2Y receptor activation generates an increase in the proportion of calcium impermeable AMPARs in OPCs.

ATP has previously been shown to increase the intracellular Ca^{2+} in NG2+ OPCs (Hamilton et al., 20101). To determine whether an increase in intracellular Ca^{2+} is involved in P2Y activated AMPAR plasticity, in OPCs, cells were treated with 20μM BAPTA-AM + ATP. In these conditions the effects of ATP were significantly reduced (RI BAPTA-AM + ATP = 0.55 ± 0.05; n = 6; P = 0.45 compared to control; n = 6 P = 0.9) (**Figure 4.6D**) suggesting that an increase in intracellular Ca^{2+} is involved in the P2Y induced AMPAR plasticity.

We next examined whether P2Y mediated plasticity is dependent on protein synthesis. OPCs were treated with C-hex (25μM) and 1mM ATP and AMPA rectification measured. In contrast with mGluR dependent change, ATP mediated AMPAR plasticity was not dependent on C-hex (C-

hex + ATP RI = 0.82 ± 0.05; P = 0.019 compared to control RI; n=5). These results suggest that AMPAR plasticity mediated through mGluR and P2Y receptors involve different intracellular pathways.

As described above, P2Y activation produces an increase in GluA2 containing AMPAR. This would therefore be expected to produce a decrease in single-channel conductance of AMPARs in OPCs. The effects of ATP on AMPAR channel properties in OPCs were examined. Cells were treated with 1mM ATP for 10minutes at room temperature, and single channel properties were analyzed from control and ATP treated outside-out patches. ATP significantly decreased the AMPAR single-channel conductance from, control cells 35.65 ± 3pS n = 8 to ATP treated cells 17.37 ± 2.4pS n = 7 (P = 0.002) **(Figure 4.7C).** This decrease in channel conductance is further evidence of an increase in calcium impermeable AMPARs. Interestingly the desensitization kinetics of AMPARs from ATP treated were significantly slower when compared with control AMPARs (control τ_{des} = 4.8 ± 0.4ms, ATP τ_{des} = 6.8 ± 0.6ms; P = 0.01), as might be expected for an increased incorporation of GluA2 containing AMPARs receptors (Oh et al., 2006) **(Figure 4.6E).**

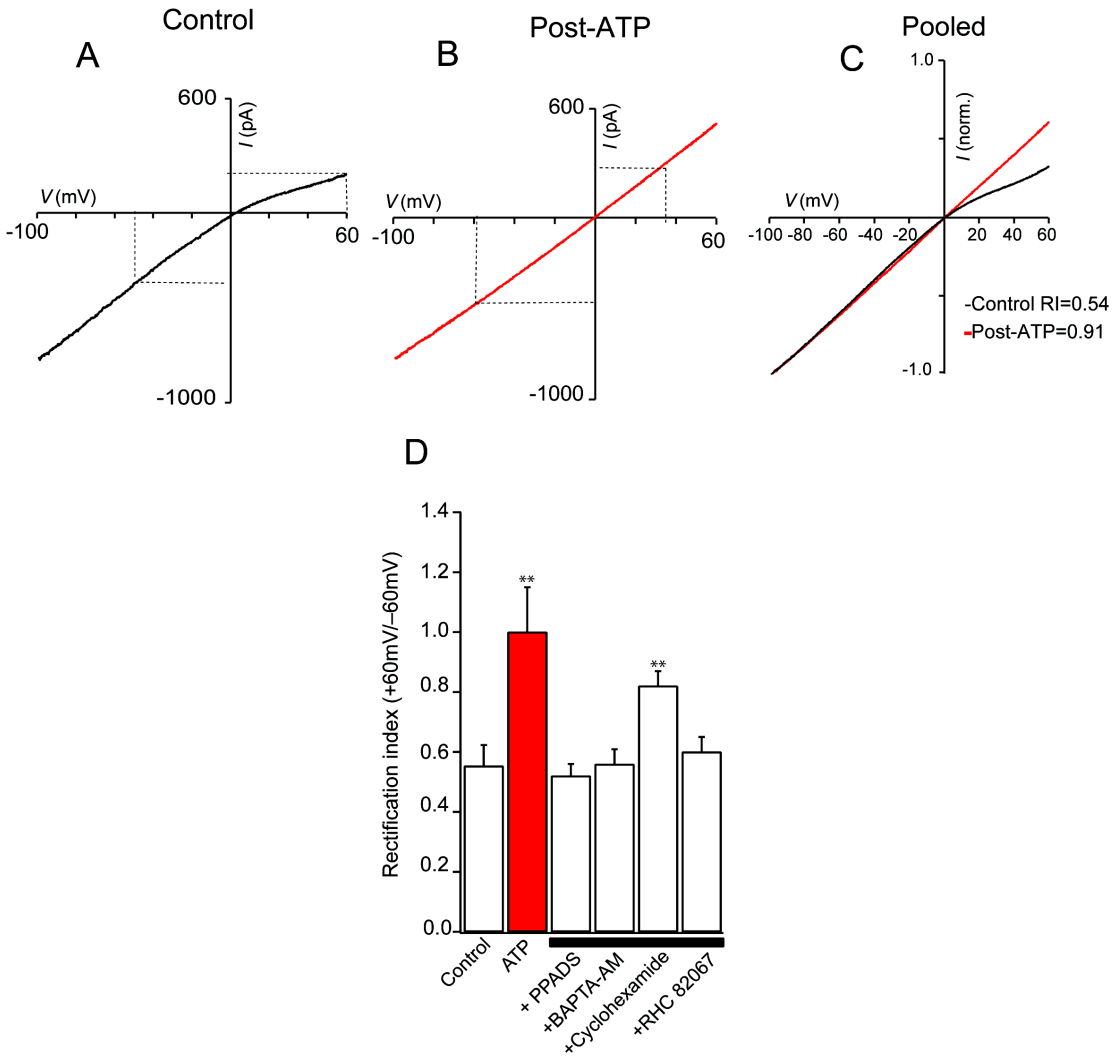


Figure 4.6. ATP regulates AMPARs properties in optic nerve OPCs.

(**A-C**) Whole cell I-V plots and averaged I-V plots obtained in response to 100μM glutamate (+60/-100) applied to cells bathed in either in control external solution or in external solution containing 1mM ATP for 10minutes at 25^0C. Activation of P2 receptor increased AMPAR rectification index from control cells 0.55 ± 0.07 to ATP treated cells R1= 0.911 ± 0.1; P = 0.005**; n = 6 and n = 8. **(D)** The increase in AMPAR RI response produced by ATP were reduced with the addition of either P2 antagonist PPADS, the phospholipase C inhibitor RHC 82067 and the calcium chelator BAPTA-AM (RI = PPADS+ATP = 0.52 ± 0.04 n = 5; P = 0.7 compared to control RI; RHC-82067 RI = 0.63 ± 0.03; P = 0.47 compared to control; n = 5; BAPTA-AM RI = 0.55 ± 0.05; n = 6; P = 0.9 compared to control). Cyclohexamide did not effect ATP mediated AMPAR plasticity in OPCs (C-hex + ATP RI = 0.82 ± 0.05; P=0.019* compared to control RI; n=5). Kruskal-Wallis rank sum test followed by pair wise Wilcoxon rank sum tests with Holm's sequential Bonferroni correction

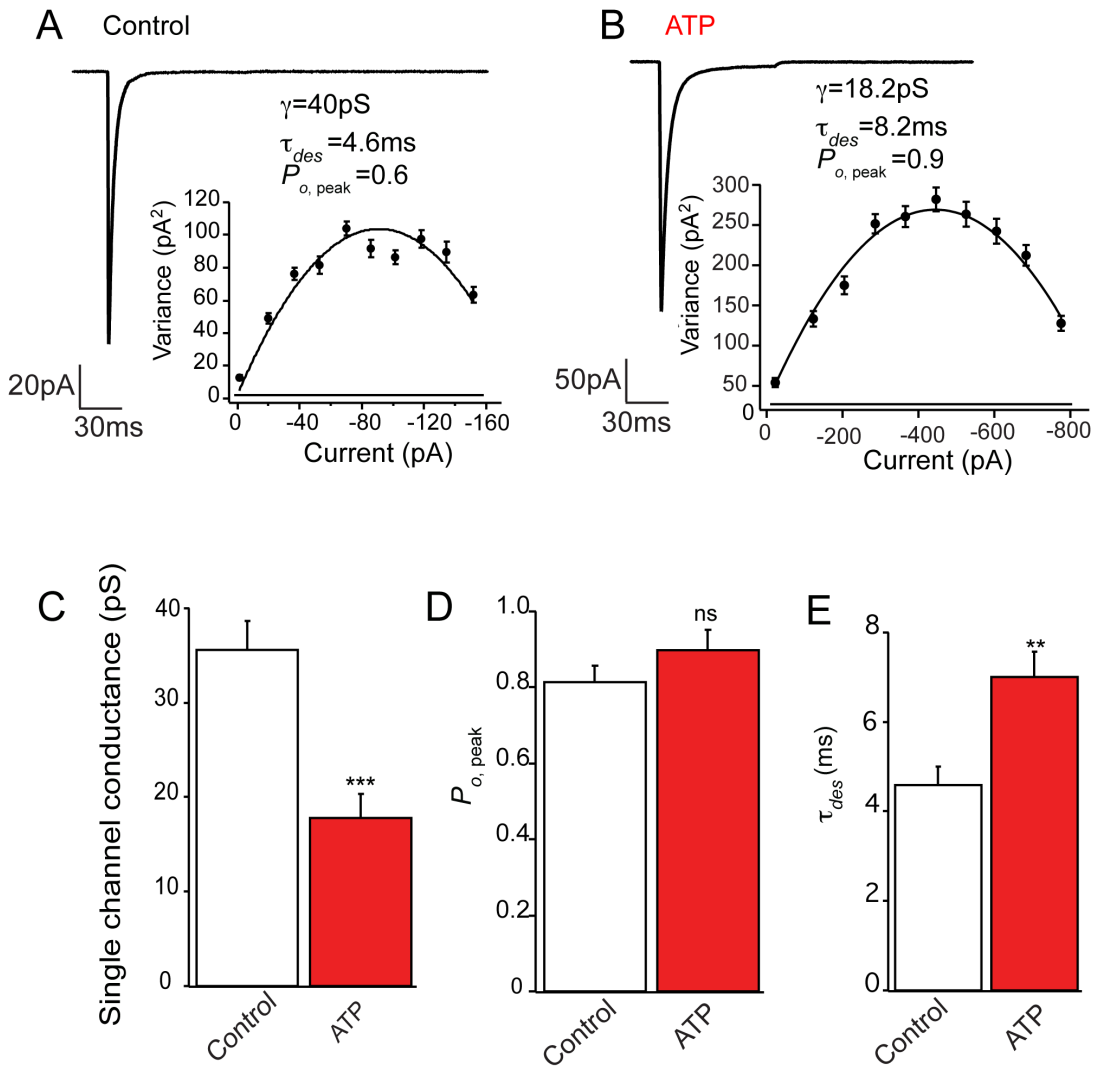


Figure 4.7. Purinergic receptor activation alters AMPAR channel properties in OPCs.

(A-B) Average responses to fast application of 10mM glutamate (100ms, -60mV) to outside-out patches. Control current is the mean of 88 traces; ATP treated current is the mean of 40 traces. Insets show plots of noise variance vs. inward current. **(C)** The estimated mean channel conductance was significantly decreased with the application of 1mM ATP, conductance of control cells 35.65 ± 3pS n = 8 reduced to 17.37 ± 2.4pS following ATP treatment (n = 7; P = 0.002**) (unpaired t-test). **(D)** The $P_{o,peak}$ of the AMPARs remained unaltered after treatment with ATP (Control $P_{o,peak}$ 0.8 ± 0.07, ATP 0.9 ± 0.06) (ns). **(E)** ATP increased AMPAR desensitization (control τ_{des} = 4.8 ± 0.4ms, ATP τ_{des} = 6.8 ± 0.6ms) (P = 0.005**) (unpaired t-test).

4.3.7. OPCs express TARPs

Previous studies have identified TARPs as important proteins in controlling the properties of AMPARs involved in hippocampal plasticity (Rouach et al., 2005; Tomita et al., 2005b). However was unknown whether TARPs are expressed in OPCs, and whether they are required for mGluR mediated AMPA receptor plasticity in these cells. To identify the possible TARPs expressed in optic nerve OPCs, the mRNA was extracted from the optic nerves of P7 rats and RT-PCR were carried out using primers for TARPs (γ-2, γ-3, γ-4, γ-5, γ-7 and γ-8) and the TARP related proteins γ-1 and γ-6. We detected γ-2, γ-3, γ-4, γ-5 and γ-6, (**Figure 4.8 A**).

To further identify which TARPs are present in OPCs, cells were labeled with anti-γ-2/γ-8 (pan-TARP), anti-γ-5 and anti-γ-7 antibodies, however they did not label with γ-5 or γ-7 antibodies (**Figure 4.8 B**). It is possible that the mRNA detected for γ-4 and γ-5 is produced from GFAP positive astrocytes present in the optic nerve. It is also plausible that during differentiation of OPC into premeylinating cells and eventually into oligodendrocytes the expression levels of TARPs could alter. To address this OPCs were treated with thyroxine to induce differentiation into mature OPCs (identified with the antibody against the cell surface antigen O4) and into oligodendrocytes (identified with the an antibody against the cell surface antigen only expressed in oligodendrocytes O1). These cells were treated with anti-γ-5 ant-γ-7 and anti-γ-2-/γ-8. Premyelinating OPCs labeled with O4 and γ-2/γ-8, and similar to immature OPCs did not label with anti-γ-5 or with anti-γ-7, however there were reduced expression levels for γ-2/γ-8 in oligodendrocytes (**Figure 4.8 C-D**).

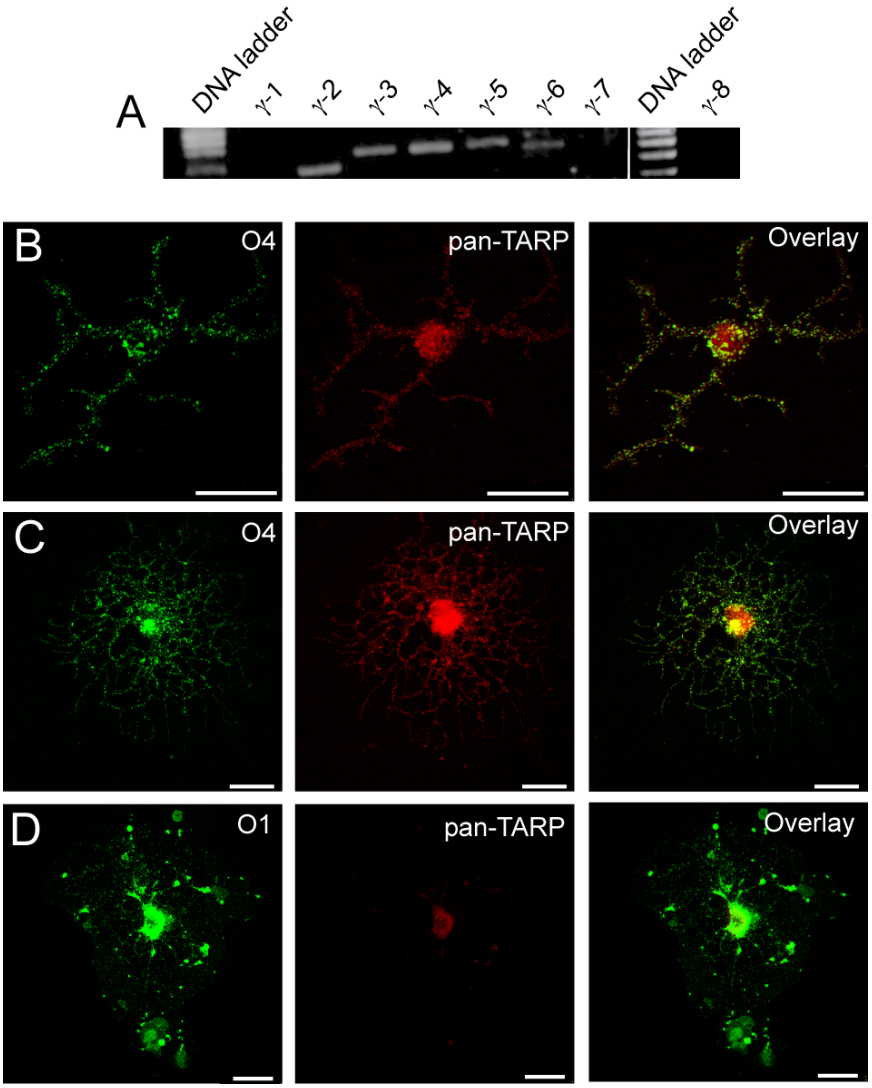


Figure 4.8. Immature and differentiated optic OPCs express TARPs.

(A) TARP cDNA expression pattern in rat optic nerve. γ-2 is highly expressed with a faint expression for γ-3 and the TARP related protein γ-6 n = 3. **(B)** Confocal image displaying typical example optic nerve OPC labeled with anti-γ2/γ8 (red) antibody and O4 (green). Cells labeled with the antibody against O4 and γ-2/γ-8 **(C)** Example of a premyelinating optic nerve OPCs labeled with anti-γ2/γ8 and O4**. (D)** Optic nerve oligodendrocytes labeled with marker O1+ showed reduced expression of TARP compared with pre-myelinating cells (n = 20 OPCs; n = 15 premyelinating OPCs; n = 21 oligodendrocytes) (Scale 25μm).

4.3.8. TARPs control AMPAR channel conductance in OPC

The TARP γ-2 has been shown to increase AMPAR channel conductance in expression systems (**Figure 2A chapter 3**). The PDZ domain of γ-2 is critical for the interaction between TARPs and the synaptic protein PSD-95 (Bats et al., 2007). Chen et al., 2000 demonstrated that in granule cells from stargazer mice it is possible to rescue surface AMPARs by transfecting cells with γ-2. In contrast, transfecting these cells with a mutant construct of γ-2, that lacked the 'TTPV' PDZ binding domain, failed to rescue the AMPARs responses.

Is γ-2 required to control functional properties of surface AMPAR in OPCs? To address this, cells were transfected with either GFP control or with a mutant γ-2 construct (GFP γ-2Δ308) that is lacking in the 'TTPV' PDZ binding domain. The AMPAR channel properties in these cells were examined from glutamate evoked responses from excised patches. The AMPAR channel conductance was significantly reduced in γ-2Δ308 transfected cells compared with control GFP transfected OPCs (control singe channel conductance = 39 ± 5pS to conductance of 21 ± 1pS for γ-2Δ308 transfected cells; n=6 and n = 6; P = 0.007**. The AMPAR channel desensitization properties remained unchanged between GFP and the γ-2 Δ308 transfected cells (control 5.2 ± 0.33ms, γ-2Δ308 5.1 ± 0.6ms). These results suggest that the -TTPV PDZ domain of γ-2 is critical for regulating the AMPAR in OPCs. It seems possible that the reduction in the single-channel conductance could be attributed to altered trafficking and cell surface expression of CP-AMPARs in OPCs, to explore this I will be next

addressing the rectification properties in OPCs expressing TTPV mutant γ-2.

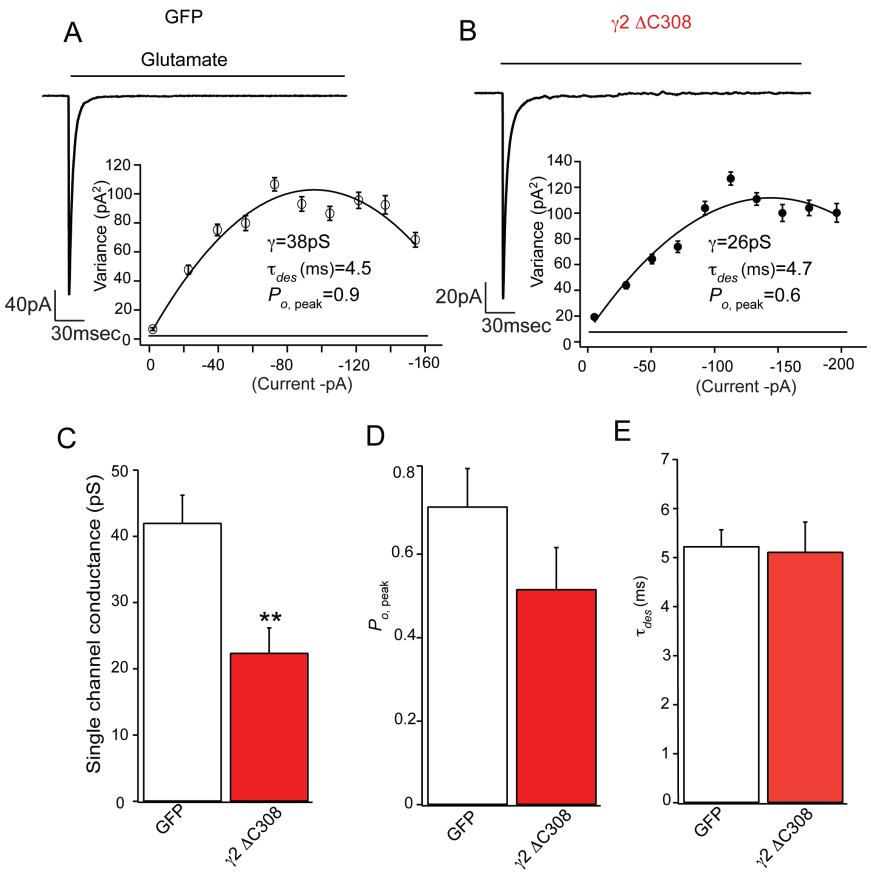


Figure 4.9. Surface expression of functional CP-AMPARs in optic OPCs is dependent on the C-tail of γ-2.

(**A-C**) OPCs were transfected with GFP-γ-2, GFP γ-2 308 ΔC308. Current variance traces from OPC transfected with GFP (mean of 90 traces) and GFP-γ2 ΔC308 (mean of 52 traces) were obtained from outside-out patches of rapid application of 10mM glutamate (100ms, -60mV). (**D**) The overexpression of γ-2 Δ308 produced a significant reduction in single-channel conductance to 21 ± 1pS (n = 6) (P=0.007** unpaired t-test). **(E)** γ-2Δ308 does not alter AMPA channel desensitization from control 5.2 ± 0.33ms to γ-2 γ-2Δ308 truncation 5.1 ± 0.6ms (ns unpaired t-test).

4.3.9. TARP γ-2 regulates mGluR plasticity

As the deletion of the C-terminus of γ-2 in OPC produced a reduction in single-channel conductance of AMPARs is consistent with a loss of CP-AMPARs, it was possible that when group I mGluRs are activated in OPCs, γ-2 maybe is responsible for the delivery of CP-AMPARs to the cell surface.

To determine whether γ-2 was controlling the trafficking of CP-AMPARs in subunit specific manner I transfected OPCs with either full-length γ-2 in a GFP vector or with γ-2ΔC308 mutant also in a GFP vector. The expression of the γ-2Δ308 mutant significantly increased the AMPAR rectification index compared with that of OPCs transfected with full-length γ-2 (γ-2 wild-type RI= 0.5 ± 0.03, γ-2Δ308 RI= 1.1 ± 0.05; P = 0.001) (**Figure 4.12**). These results suggest that γ-2 can participate in regulating the trafficking of CP-AMPARs in OPCs.

Such a deficiency in the delivery of CP-AMPAR to the membrane in OPCs expressing γ–2Δ308 could affect mGluR AMPAR plasticity. To investigate this possibility, γ-2Δ308 transfected cells were stimulated with DHPG. Cells transfected with γ-2Δ308 did not show a decrease in AMPAR rectification in response to mGluR activation (γ-2Δ308 RI = 1.1±0.05 γ-2Δ308+DHPG RI= 1.0±0.08), providing evidence that full-length γ-2 is required for mGluR dependent AMPAR plasticity in OPCs.

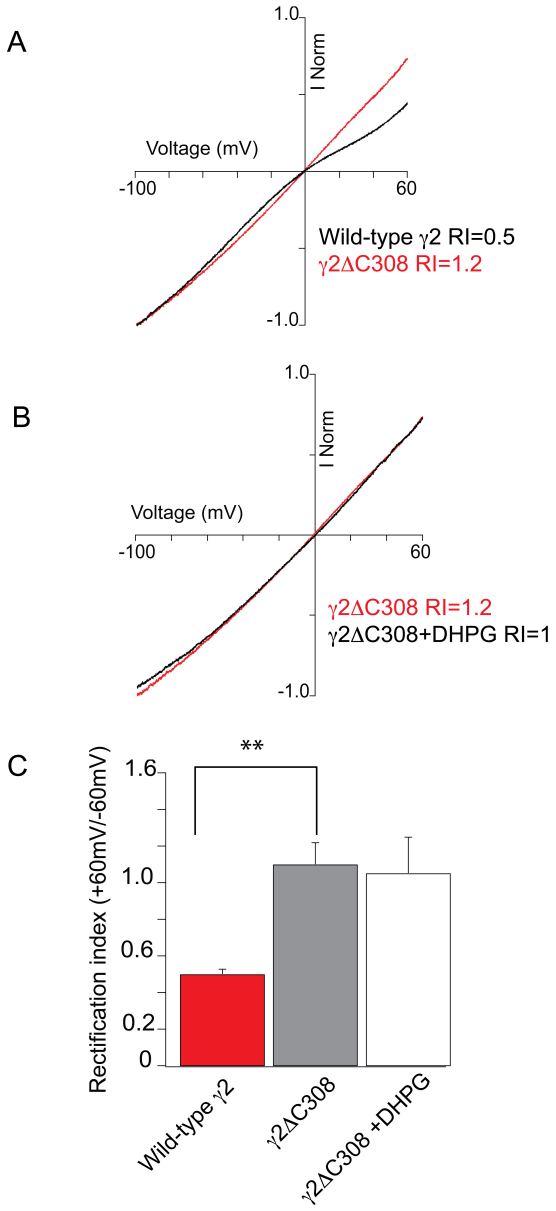


Figure 4.10. Changes in CP-AMPAR surface expression involves the C-terminus of γ−2.

(A-B) OPCs were transfected GFP-γ2 or GFP-γ2 (ΔC308). Whole-cell ramp *I-V* plots (+60/-100) were obtained from γ-2 overexpressing and expression of γ2 ΔC308 mutant cells in response to 100μM glutamate. The expression of the C-terminal mutant of γ2 significantly increased the AMPAR RI = 0.5 ± 0.03 in γ2 overexpressed cells, to RI γ2 ΔC308 = 1.1±0.05 (unpaired t-test P<0.001**). **(C)** Treatment with 100μM DHPG for 30 minutes no longer triggered an increase in rectification in cells transfected with the C-tail truncated mutation. In OPCs transfected with γ-2 ΔC308 RI=1.1±0.05 in control and 1.0±0.04 following DHPG treatment) (not significant; unpaired t-test).

4.3.10. Neuronal calcium sensor proteins co-localise with TARPs in OPCs

Calcium sensing proteins have been found to control mGluR plasticity and facilitate synaptic transmission in neurons. The neuronal calcium sensor protein 1 (NCS-1) is a homolog of the Drosophila protein frequenin, and has recently been shown to control mGluR-LTD. A study by Jo et al., 2008 demonstrated that mGluR5 activation triggers an increase in intracellular Ca^{2+}, in neurons activating NCS-1, which subsequently binds directly to PICK-1 and forms a complex with AMPA receptors, triggering internalisation (Jo et al., 2008).

As γ-2 is important for mGluR mediated AMPA plasticity in OPCs it was possible that an increase in intracellular Ca^{2+} could also trigger calcium-sensing proteins, which could regulate mGluR plasticity OPCs. To explore this possibility whether OPCs expressed the neuronal calcium sensor NCS, we initially treated cells with a specific antibody against NCS-1. Surprisingly we found high levels of the NCS-1 a protein in OPCs previously thought to be localized only in neurons. Furthermore, there was a high degree of a co-localisation of NCS-1 and γ-2 (**Figure 4.11**). This finding demonstrates that in many respects the mechanisms that control mGluR plasticity in neurons are similar in OPCs.

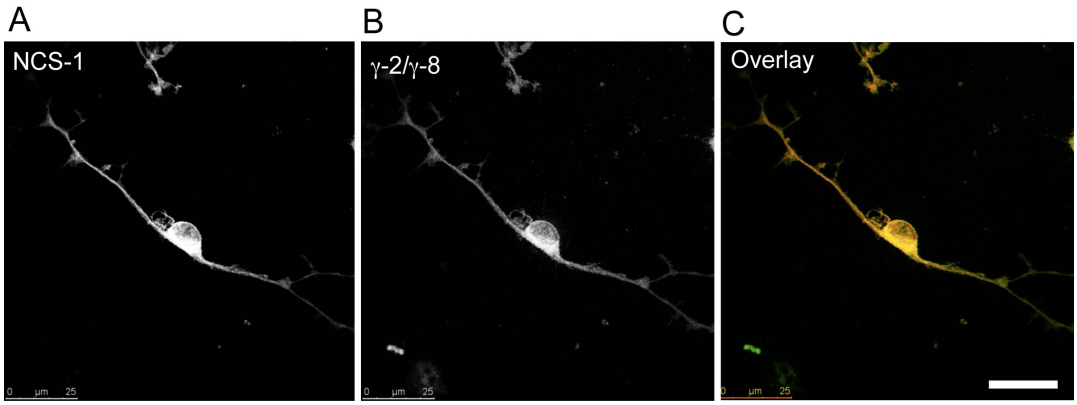


Figure 4.11. Neuronal calcium sensing (NCS-1) protein 1 is present in OPCs and colocalises with γ-2.

(A-B) Optic nerve OPCs stained with anti-NCS-1 and anti-γ2/γ8. There was an 80% colocalisation between NCS-1 and TARPs (n=10).

4.4. Discussion

The results described in this chapter suggest that the properties of AMPARs in OPCs can be regulated by activation of group 1 metabotropic receptors (mGluR5) and ionotropic purinergic receptors (P2Y). The experiments demonstrated that stimulation of group I mGluRs produce an *increase* in the relative proportion of CP-AMPARs in the surface membrane of OPCs, while in contrast activation of P2Y receptors *decrease* relative proportion of CP-AMPARs. Various intracellular pathways have been implicated in the mGluRs mediated AMPAR plasticity previously described in neurons (Kelly et al., 2009). In the present study we found certain key intracellular pathways may also be important in the regulation of AMPAR plasticity in OPCS. Interestingly, we also demonstrate that the TARP γ-2 plays a key role in this plasticity in glia.

4.4.1. Possible physiological implications of mGluR mediated AMPAR plasticity in OPCs

The present experiments provide evidence that mGluR activation can mediate a form of AMPAR plasticity that results in an increased insertion of CP-AMPARs in the surface of OPCs. Is this likely to be of physiological or pathological relevance in the CNS? It has been demonstrated that release of glutamate from axons and also from certain glial cells (astrocytes) is a major source of the transmitter in the white matter (Kukley et al., 2007). mGluR activation and an associated increase in calcium permeable AMPARs could therefore occur in OPCs *in situ* and potentially influence their normal proliferation and differentiation and may affect OPC survival following white matter damage.

Furthermore, recent evidence suggests that evoked release of transmitter (glutamate) from nerve terminals in the hippocampus onto OPCs causes a switch from calcium impermeable CI- to CP-AMPARs in these cells (Ge et al., 2006).

Although the mechanism underlying this change was not previously explored in detail, our results would strongly suggest that it is likely to involve activation of mGluRs. It is of particular interest the mGluR activation alters the proportion of CP-AMPARs in neurons, but invariably results in a loss (rather than an increase) in CP-AMPARs (Mameli et al., 2007;Kelly et al., 2009). Why does mGluR activation differ in its effect on AMPAR plasticity in OPCs and interneurons? Interneurons in the cerebellum and VTA express multiple TARPs including γ-2 and γ-7; they also express the postsynaptic density protein PSD-95. However, OPCs appear to express only γ-2 and SAP-97 (Kato et al., 2007). Therefore, a potential difference in the mGluR plasticity between neurons and glia could be attributed to γ-7. The TARP γ-7 has been shown to have a unique C-terminal domain making it is possible that it is interacting with a variety of different trafficking proteins (Deng et al., 2006). It is also possible that activation of mGluR1 in neurons can phosphorylate the C-terminus of γ-7 resulting in an increased insertion of GluA2 containing AMPARs to the surface of neurons. In contrast, in OPCs stargazin may preferentially increase the expression of GluA4/GluA3 containing AMPARs to the surface. It would therefore be of interest to transfect OPCs with γ-7 and observe whether the mGluR dependent AMPAR plasticity produced is similar to the plasticity seen in neurons.

During development immature OPCs are expressed throughout the CNS and are highly proliferative prior to forming myelinating oligodendrocytes (Bogler et al., 1990; Raff et al., 1988). Factors that regulate their migration, and differentiation of immature OPCs into oligodendrocytes, have been the focus of numerous studies, which have identified activation of AMPARs as a factor that can regulate the differentiation of OPCs into myelinating cells (Yuan et al., 1998). Thus, treating cerebellar slices with AMPAR agonists decreases both the proliferation and the number of OPCs (Yuan et al). However, the signaling mechanism that

controls the anti-proliferative properties of glutamate has not been explored in any detail. It is possible that the activation of mGluRs in OPCs may be a contributing factor in the ability of glutamate to halt OPC proliferation.

It is also of note that the effect of mGluR activation on the proliferative properties of OPC can be important following white matter damage, after brain trauma, where there is evidence that OPCs are attempting to remyelinate neurons (Carroll et al., 1998). The increased release of glutamate due to tissue damage during white matter damage can 'overactivate' mGluRs in OPCs, potentially reducing the ability of OPCs to re-populate the demeylinating plaque (Rothman and Olney, 1986).

OPCs are derived from early progenitor cell (O2A progenitors). O2A progenitors have high expression levels of mGluR1 and mGluR5; these cells also express mixtures of calcium impermeable and CP-AMPARs. Activation of mGluR1/5 has been shown to protect O2A progenitors from cell damage caused by exposure to high levels of kainate and staurosporin (Deng et al., 2004; Luyt et al., 2006). This neuroprotective effect of mGluR1/5 could be due to activation of serine/threonine kinase Akt, which is important for neuronal cell survival (Datta et al., 1999). However, there is evidence that as O2A progenitors differentiate into OPCs, there is a decrease in the expression of mGluR1 with only low levels of mGluR5 remaining (Deng et al., 2004). An increased calcium influx through CP-AMPARs has been linked to increased cell death of OPCs (Alberdi et al., 2002). The reduced expression of mGluR1 during differentiation of O2A progenitors can lead to an increased susceptibility of immature OPCs to cell death during ischemia, as the activation of remaining mGluR5 could still result in an increase in CP-AMPARs, without the associated increased activation of the protective Akt pathway provided by mGluR1.

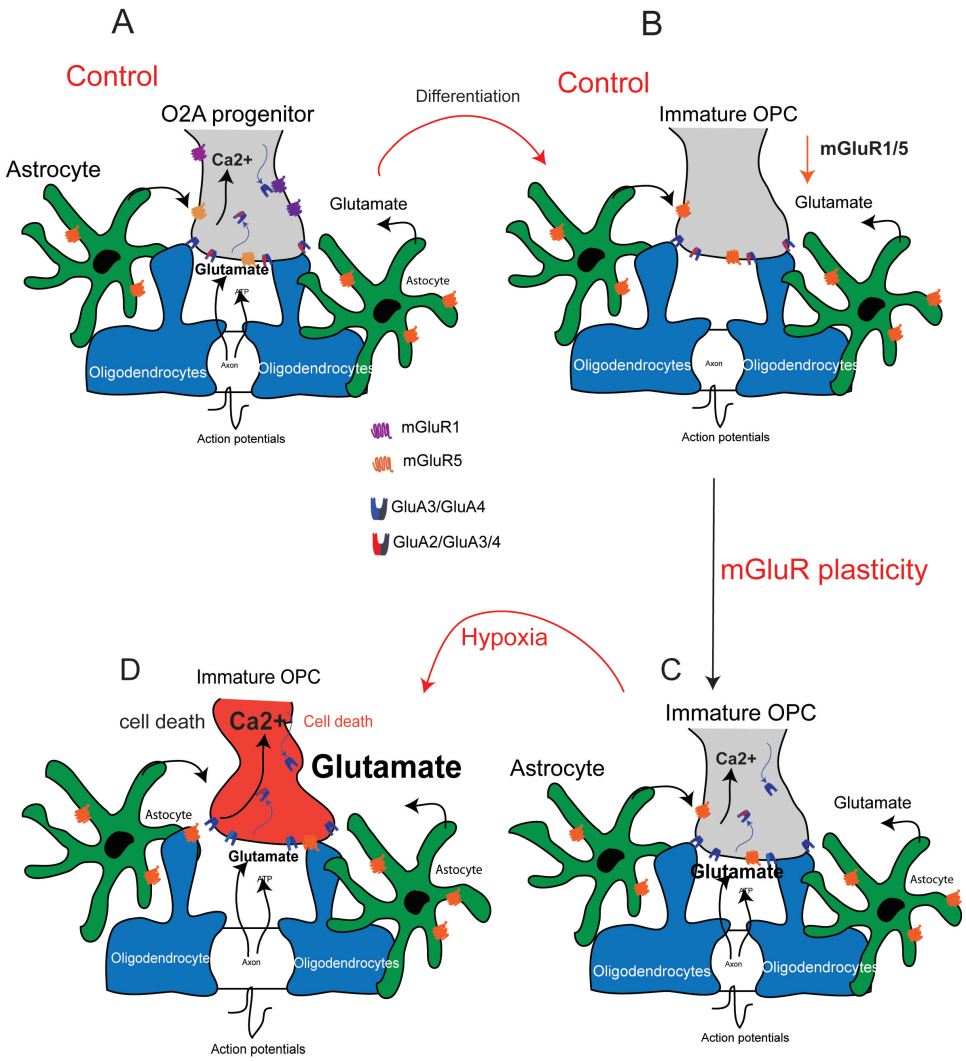


Figure 4.4.1.2. Hypothetical model of mechanisms contributing to OPC cell death.

(A) O2A progenitors express high levels of mGluR1, mGluR5 and CI and CP-AMPARs. The activation of mGluR1/5 in O2A progenitors may have a neuroprotective effect as it initiates the Akt pathway**. (B)** During the differentiation of O2A progenitors into OPCs there is a loss of mGluR1 and reduced expression levels of mGluR5; however, these cells still express CP-AMPARs**. (C)** Immature OPCs undergo mGluR mediated AMPAR plasticity after the release of glutamate from axons and astrocytes, resulting in an increased proportion of CP-AMPARs at the surface**. (D)** Excessive release of glutamate during hypoxia could activate the remaining mGluR5 that promote the insertion of CP-AMPARs in OPCs, thus leading to cell death.

4.4.2. ATP produces a novel plasticity in OPCs

The present experiments also demonstrate that the neurotransmitter ATP produced a form of AMPAR plasticity that results in replacement of CP-AMPARs by CI-AMPARs in OPCs. ATP is known to be released from axons and astrocytes in the brain (including optic nerve) thus resulting in increased intracellular levels of $[Ca^{2+}]i$ in OPCs (Hamilton et al., 2010; Ishibashi et al., 2006).

ATP is a relatively unusual transmitter as it is rapidly degraded into adenosine, which acts on another group of G-protein, coupled receptors A1, A2 and A3 (adenosine receptors). Adenosine has been shown to activate OPC differentiation in myelinating oligodendrocytes (Stevens et al., 2002). In the present study we found that mature premyelinating OPCs have reduced expression of CP-AMPARs. It is possible that *in vivo* ATP can prime OPC for differentiation into myelinating cells by reducing expression of CP-AMPARs and promoting differentiation *via* activation of the adenosine pathway. However, in a recent study it was proposed that pre-myelinating OPCs receive fewer glutamatergic inputs from neurons, which may argue against this. Nevertheless, it is possible that the levels of ATP released from astrocytes and axons may remain constant during development, which could be a potential mechanism for controlling OPC differentiation and for the reduced expression of CI-AMPARs during development (Kukley et al., 2010).

What are the reasons for the reduced expression of CP-AMPARs during differentiation of OPCs into oligodendrocytes? Pende et al demonstrated that increased calcium influx through AMPARs increases the expression of NGFI-A and c-fos, which are markers for elevated protein expression (Pende et al., 1994). It is possible that OPCs require Ca^{2+} influx through CP-AMPARs early in development to allow them to migrate to nerve axons and differentiate into

myelinating cells (Gudz et al., 2006). However, once OPCs have differentiated, nerve terminals will require oligodendrocytes to become stationary. It may thus be beneficial for the expression of CP-AMPARs to be reduced in myelinating oligodendrocytes.

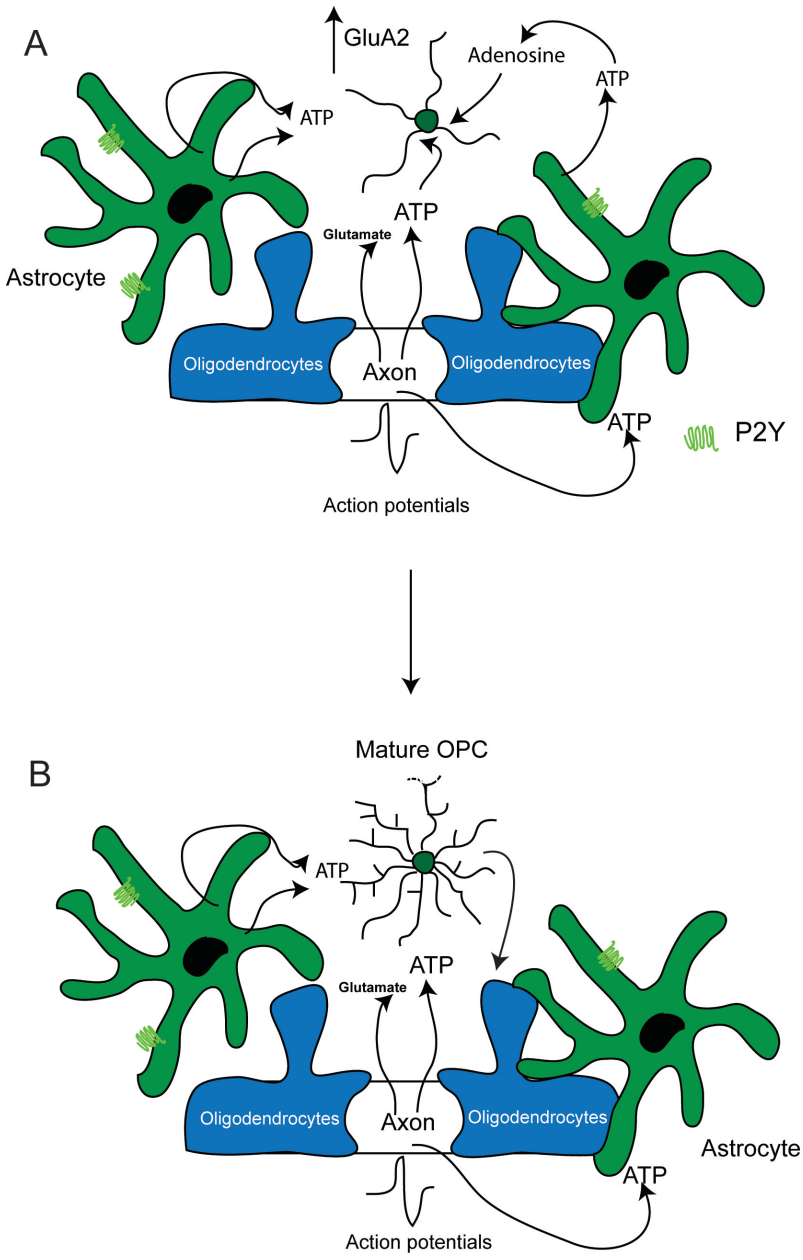


Figure 4.4.1.3. Hypothetical model of ATP regulation of the maturation of OPCs.

(A) ATP released from axons and astrocytes may activate P2Y receptors expressed in OPCs, leading to a reduced proportion of CP-AMPARs **(B)** ATP may then be rapidly degraded into adenosine, resulting in differentiation of OPCs into oligodendrocytes.

4.4.3. Key intracellular kinases control glial AMPAR plasticity in OPCs

mGluR1 mediated AMPAR plasticity in VTA neurons and cerebellar interneurons are thought to be regulated by the PI3K-Akt-mTOR pathway. We found that mGluR5 dependent AMPAR plasticity in OPCs is also regulated by PI3-kinase and protein synthesis. However, in contrast with the situation in neurons, in OPCs activation of mGluRs *increases* the proportion of CP-AMPARs. It seems possible that activation of mGluR5 in OPCs could result in the activation of several as yet unidentified auxiliary proteins that preferentially traffic CP-AMPARs. The activation of CP-AMPARs in O2A progenitors has been shown to increase levels of c-jun and activate early immediate genes (Pende et al., 1994). In the current study we identify that c-jun N-terminal kinases can control mGluR plasticity in OPCs. It is likely that activation of mGluRs, together with activation of CP-AMPARs, acts to increase mRNA expression, promoting increased expression levels of CP-AMPARs in the surface of OPCs.

Activation of mGluRs in neurons triggers the activation of the protein Homer, increasing intracellular calcium. This rise in intracellular calcium is believed to activate PICK-1, thereby initiating internalization of AMPARs. It was previously unknown whether PICK-1 regulates AMPARs in glial cells. We find compelling evidence that PICK-1 is important in regulating internalization of GluA2 containing AMPARs from the surface of OPCs.

4.4.4. TARPs play a unique role in OPCs

Previous studies have shown that AMPAR channel conductance in Bergmann glial cells is controlled by the TARP γ-5. The TARPs that regulated AMPARs in

OPCs and oligodendrocytes were unknown. In the present study we find that stargazin is expressed in OPCs and is important in AMPAR regulation.

Firstly, we identified that γ-2 influences the single-channel conductance of AMPARs in OPCs. Importantly we find that the –TTPV PDZ binding domain of γ-2 is critical for its ability to control the trafficking of CP-AMPARs in the surface of OPCs, as previously seen in cerebellar granule cells (Chen et al., 2000). Secondly, we found that γ-2 regulates mGluR mediated AMPAR plasticity in OPCs. Previous studies have shown that phosphorylation of serine residues in the C-terminus of γ-2 can regulate neuronal LTP and LTD (Tomita et al., 2005). Thus, a potential model for the role of γ-2 in OPC mGluR plasticity is that the increased influx of intracellular calcium can lead to the activation of protein kinases, which in turn phosphorylate the C-terminus of γ-2. Increased phosphorylation of the C-terminus of γ-2 could enhance the ability of γ-2 to interact with PDZ binding proteins, resulting in increased delivery and stabilization of CP-AMPARs in the surface of OPCs. Previous work has also demonstrated that early in development the phosphorylation by PKA of Ser-482 in GluA4 is necessary and sufficient for GluA4-containing AMPARs to be delivered to neuronal synapses (Correia et al., 2003). It is possible that in OPCs the activation of mGluR may also increase the phosphorylation of GluA4 resulting in increased association with γ-2, thereby leading to a preferential increase in stability of GluA4 containing AMPARs at the surface of OPCs.

Chapter 5. Cornichon proteins CNIH2/3 are expressed on the cell surface of oligodendrocyte precursor cells and regulate the properties of AMPARs

5.1. Summary

As described in chapter 4, TARPs have been demonstrated to control functional properties of AMPARs. Recently, a new family of AMPAR interacting proteins was identified. The cornichons, CNIH2 and CNIH3 are a family of transmembrane proteins that appear to act as AMPAR auxiliary proteins. In a previous study CNIH2/3 expression has been identified in neurons and glia of the cerebellum and in hippocampal neurons (Lien et al., 2007; Schwenk et al., 2009). CNIH2 and 3 show some apparent similarities to TARPs in their ability to control AMPAR properties in expression systems, regulating the trafficking and the kinetic properties of AMPARs. However, in a very recent study it has been proposed that cornichons are not able to modulate the properties of cell surface AMPARs in neurons, as 'cornichoned' receptors fail to reach the cell surface in neurons (Shi et al., 2010). Here, we have Immunofluorescence data demonstrating that cornichons are expressed in the cell surface of cerebellar glial cells and OPCs.

Furthermore, electrophysiological methods were used to examine whether cornichons can modulate the properties of cell surface AMPARs in OPCs in which CNIH3 has been overexpressed. In these conditions CNIH3 significantly slowed AMPAR desensitisation, without changing AMPAR rectification properties. In addition we examined whether the overexpression of CNIH3 altered the AMPAR response to kainate.

5.2. Introduction

The identification of TARPs has led to a search for other potential transmembrane AMPAR interacting proteins in the CNS. Schwenk et al., 2009 identified the cornichon-like proteins CNIH2 and CNIH3 as AMPAR interacting proteins by proteomic C-terminus analysis of native GluA2 complexes (Schwenk et al., 2009). Cornichons are a family of 4 transmembrane proteins (CNIH1, CNIH2, CNIH3 and CNIH4). The expression of tagged CNIHs in HeLa cells has previously revealed a partial overlap with the Trans Golgi network (TGN) marker GM130. In addition to their presence in the TGN, CNIHs also partially colocalize with the endogenous COPII vesicle coat complexes that are present in the endoplasmic reticulum (ER) exit sites (Castillon et al., 2009; Castro et al., 2007). These results are suggestive of a role for CNIHs in chaperoning proteins through the ER to the TGN.

Schwenk et al*.,* 2009 used an antibody raised against CNIH2 and CNIH3 to label cells in the hippocampus and in the cerebellum. Their study revealed expression of CNIH2/3 in hippocampal pyramidal neurons, cerebellar Purkinje neurons and Bergmann glia. Furthermore, examinations using post-embedding electron-microscopy (EM) with the CNIH2/3 antibody detected CNIH2/3 in both postsynaptic and extrasynaptic sites in hippocampal cells. Biochemical experiments suggested that CNIH2 and CNIH3 interacted with native AMPARs and significantly increased the cell surface expression of GluA1 and GluA2. Finally, Schwenk and colleagues (2009) demonstrated that co-expression

of CNIH2 or CNIH3 with AMPAR in expression systems significantly slowed AMPAR deactivation and desensitisation kinetics.

By contrast, a recent study has provided evidence that CNIH2 functions as an intracellular trafficking molecule in the neurons, rather than regulating neuronal cell surface AMPARs (Shi et al., 2010). The study addressed firstly whether CNIH2 and 3 influenced AMPAR properties in HEK293T cells. They found that, like TARPs, CNIH2 increased the conductance of GluA1 containing AMPARs. However when CNIH2 was overexpressed in stargazer granule cells or in hippocampal neurons, AMPAR properties were not affected in these cell types. To compare the expression of TARPs and CNIH2, Shi et al., 2010 transfected hippocampal neurons with FLAG tagged CNIH2 and showed that it was localised in the TGN and was absent from the cell surface. This study confirmed these findings using biochemical assays. Shi et al., 2010 concluded that CNIH2 and CNIH3 do not behave as TARPs and that 'cornichoned' AMPARs never reach the plasma membrane in neurons.

Work in chapter 3 of this thesis revealed that the TARP γ-5 regulates CP-AMPARs in cerebellar Bergmann glia (Soto et al., 2009). γ-5 was previously not considered to be a TARP as it failed to rescue the AMPAR expression in stargazer cerebellar granule cells (Tomita et al., 2003). In a similar manner CNIH2 when overexpressed in granule cells cannot modify the properties of cell surface. We were therefore interested in whether CNIH2 and CNIH3 could regulate the properties of AMPARs in glia. In this study we show, using immunofluorescence and electrophysiological techniques, that like TARPs, CNIHs can modify AMPAR properties in glia.

5.3. Results

5.3.1. CNIH2/3 is expressed on the cell surface of cerebellar astrocytes

To determine whether there are differences in the levels of expression of CNIH2/3 between neurons and astrocytes in the cerebellum, the expression pattern of CNIH2/3 was studied in cerebellar neuronal-astrocytic co-cultures (P7 rats DIV7). Initially, to confirm the presence of astrocytes in these co-cultures, cells were permeabilized with 0.1% triton and labelled with anti-rabbit GFAP. The cerebellar cultures were composed of GFAP staining astrocytes, together with neurones (probably granule cells and interneurons). Permeabilized cultures were then labelled with a CNIH2/3 antibody (raised in rabbit against the entire sequence of the CNIH2 and CNIH3 proteins) and with anti-MAP2 antibody. CNIH2/3 is clearly expressed in neurons as well as in cells with astrocytic morphology (**Figure 5.1**).

The work of Shi et al., 2010 has proposed that CNIH2 is absent from the cell surface of neurons. To address the possibility that CNIH2/3 maybe present in the cell membrane of glial cells, non-permeabilized cerebellar neuronal-astrocytes were labelled with CNIH2/3. In contrast with CNIH labelling under permeabilized conditions, CNIH3 appeared to be only expressed in the surface of cells that display astrocytic morphology (**Figure 5.2**).

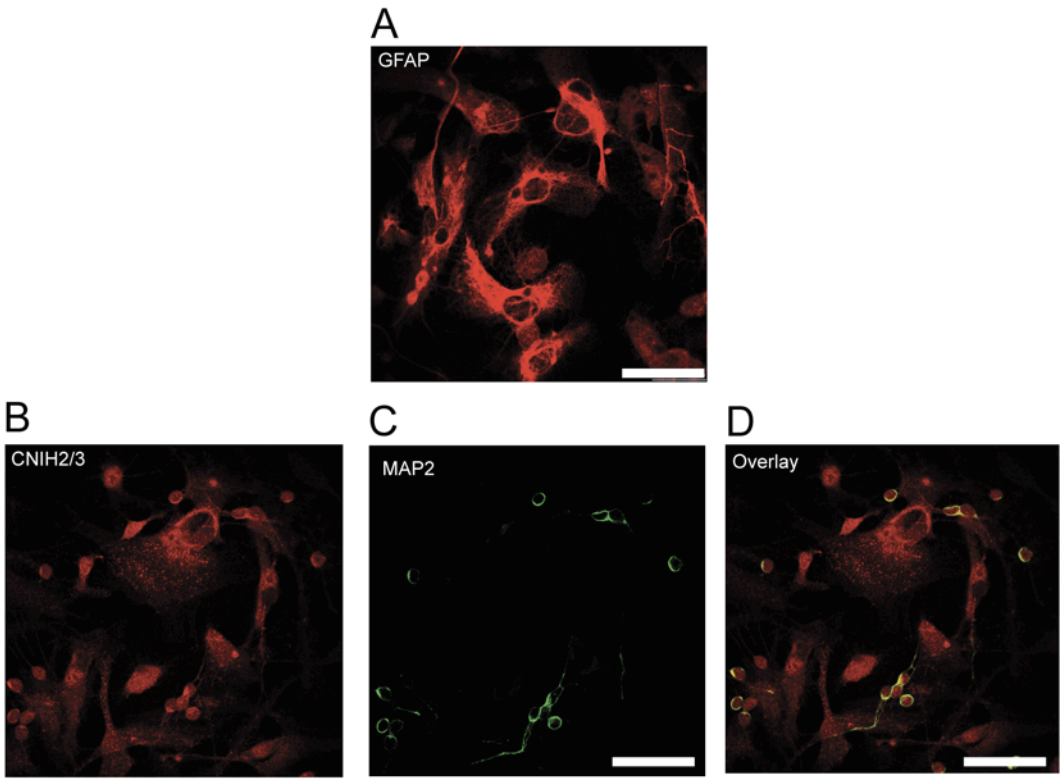


Figure 5.1. CNIH2/3 is detected in permeabilized cerebellar glia and neurons. **(A)** Cerebellar glial cells grown for 7 days in the presence of 10% FCS were stained with GFAP. **(B-D)** Mixed neuron-astrocyte cultures were fixed with 4% PFA, permeabilized with 0.1% triton and stained with rabbit anti-CNIH2/3 (red) and anti-MAP2 (green). CNIH2/3 is localised in cells showing astrocyte morphology, and in MAP2 positive neurons. (Scale bar 30 μm).

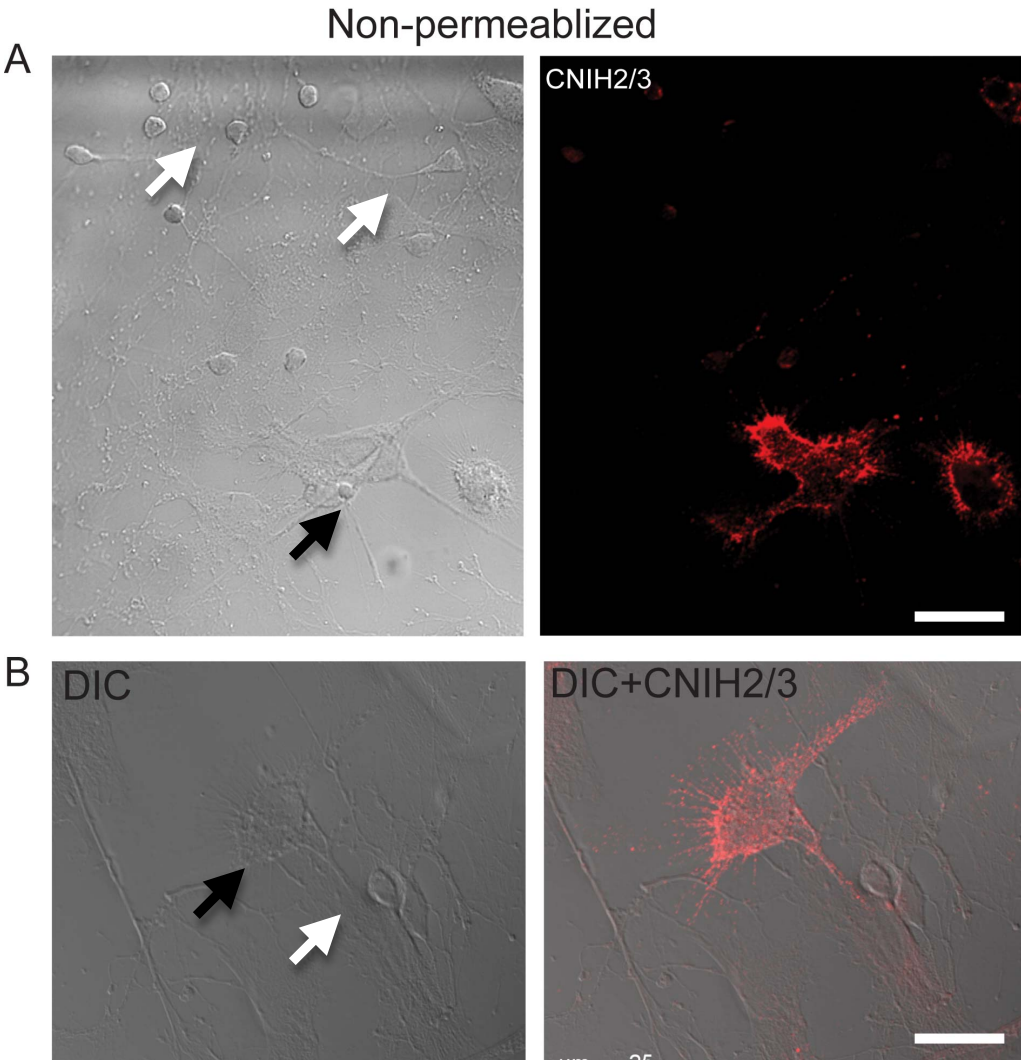


Figure 5.2. CNIH2/3 is present in the cell surface of cerebellar glia.

(**A-B**) Cerebellar neuron-glia cultures were fixed with 4% PFA and stained with anti-CNIH2/3 under non-permeabilized conditions. CNIH2/3 is highly expressed at the surface of cell glia cells (black arrow) and is expressed at lower levels in the surface of neuronal cells (white arrows)(Scale bars 25 μm).

5.3.2. CNIH2/3 is expressed in oligodendrocyte precursor cells

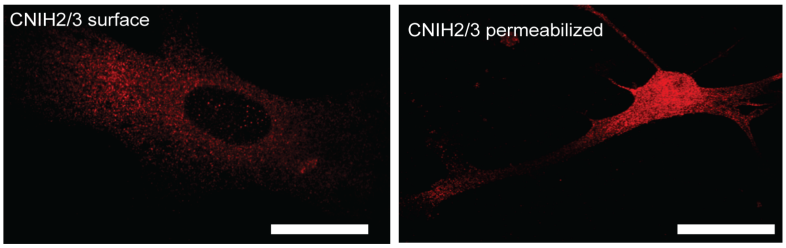
We next considered whether CNIH2/3 is expressed in other glial cells that are known to express AMPARs. Oligodendrocyte precursor cells (OPCs) are a class of glial cells that have been previously demonstrated to express a mixture of AMPAR subunits, as described in chapter 4. Investigating which proteins can regulate AMPAR properties in OPCs is of particular importance as an increase influx of Ca^{2+} through OPC CP-AMPARs has been linked to apoptosis of OPCs and white matter damage during hypoxia (Deng et al., 2003).

To address whether CNIH2/3 is also expressed in optic nerve OPCs, we initially permeabilized OPCs and labelled them with anti-CNIH2/3 and with the antibody O4 (a marker for OPCs). In common with cerebellar astrocytes, OPCs express CNIH2/3 in their cell body and along the processes (**Figure 5.3B)**. Furthermore under non-permeabilized conditions we found high levels of CNIH2/3 expression at the cell surface.

It seems possible that CNIH2/3 expression may alter during the maturation of OPCs into highly differentiated pre-oligodendrocytes (as described in chapter 4). We found that the expression levels on CNIH2/3 were on average lower in adult pre-myelinating OPCs (**Figure 5.3D**). We also examined the expression pattern of CNIH2/3 in astrocytes from the optic nerve and found that under non-permeabilized conditions cornichons were also expressed. Interestingly, the expression of CNIH2/3 was not apparent in all astrocytes examined suggesting differences in the expression levels during differentiation (**Figure 5.3A-B**). These results are suggestive that CNIH2/3 is present in the cell surface of many glial cell types, indicating a potential role in the regulation of cell surface AMPARs.

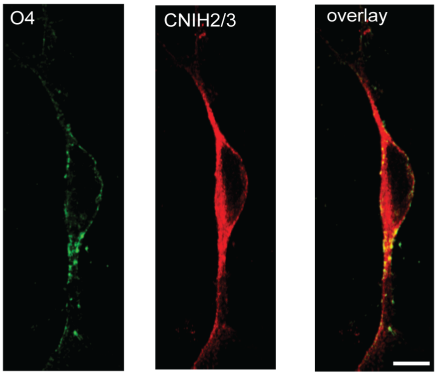
A

Optic nerve astrocyte



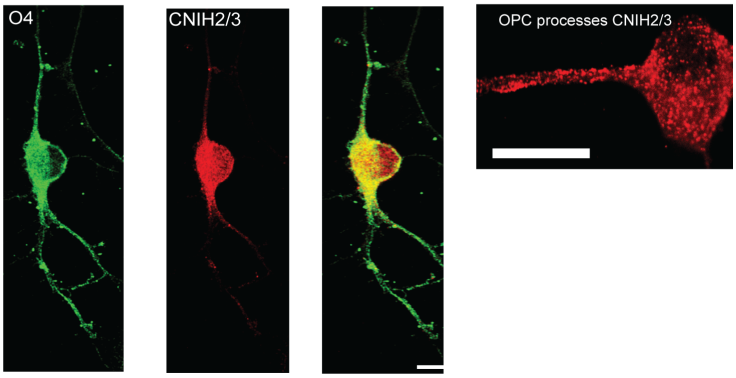
OPC permeabilized

B



OPC permeabilized

C



D

Mature Pre-Oligodendrocytes Permeabilized

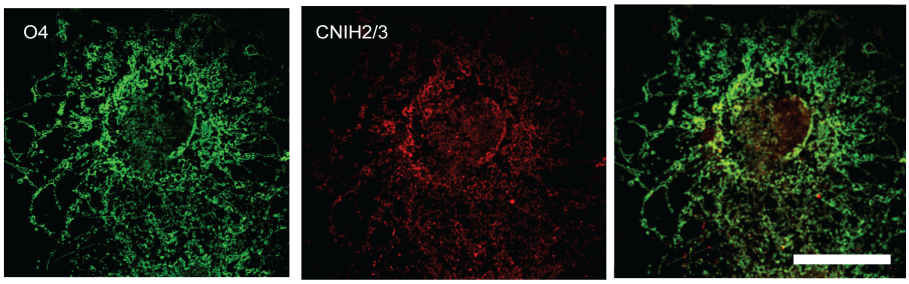


Figure 5.3. CNIH2/3 is expressed at the cell surface of optic nerve glia.

(A) Optic nerve astrocytes kept for 7-days *in vitro* were permeabilized or non-permeabilized and labelled with CNIH2/3 antibody (scale bar 25μm). **(B)** Optic nerve OPCs under non-permeabilized conditions were stained with anti-O4 and anti-CNIH2/3. CNIH2/3 is expressed at a high level in O4 positive cells (**C**) OPCs permeabilized with 0.5% triton and stained with anti-CNIH2/3 and O4 display a punctuate expression of CNIH2/3 in cell processes and cell soma (scale bar 10μm) **(D)** Mature pre-oligodendrocyte cells treated with thyroxine for 48 hours express low levels of CNIH2/3 compared to OPC (scale bar 25μm).

5.3.3. CNIH3 overexpression alters OPCs cell surface AMPAR kinetics

Next we wanted to examine whether cornichons could regulate the cell surface AMPARs on OPCs. In chapter 4, we demonstrated that the properties of cell surface AMPARs in OPCs match closely with that of TARPed AMPAR. It is therefore likely that the properties of OPC AMPARs are influenced by γ-2 rather the by cornichons. We therefore examined whether overexpression of cornichons could modify the rectification, conductance, and kinetics of AMPARs by co-overexpressing with GFP and cornichon cDNA. In this study I transfected OPCs with CNIH3 as it has a more widespread distribution in the CNS compared to CNIH2, which is highly localized to the hippocampus (Lein et al., 2007).

Initially, we examined the properties of AMPARs from control OPC using outside-out patches exposed to 10mM glutamate, and found OPC AMPARs in control conditions to be composed of a mixture of both calcium permeable and calcium impermeable AMPARs (RI +60/-80 = 0.30 ± 0.02 n = 3) (**Figure 5.4**). We then examined the properties of OPC AMPARs when overexpressed with CNIH3, and found no alterations in the rectification index (RI = 0.34 ± 0.04 n=3 P = 0.29). These results indicate that over-expression of CNIH3 did not alter the fraction of CP-AMPAR subunits. We

then examined whether CNIH3 could modify AMPAR channel conductance and kinetic properties of CNIH3 transfected OPCs. There were no alterations in the single-channel conductance of CNIH3 transfected OPCs (OPC 38 ± 3.8pS n = 7; +CNIH3 28.4 ± 3.8pS n = 6) (P = 0.09) (**Figure 5.5B**). However the effect on desensitization kinetics of CNIH3 over-expression was clear (τ_{des} control = 5.31 ± 0.54ms n = 10 to CNIH3 τ_{des} = 9.87 ± 1.39ms n = 11) (P = 0.007**) (**Figure 5.5C-F**).

This data suggests that CNIH3 is able to reach the cell surface in certain glial cells and modulate the properties of AMPARs, however it is less clear if CNIH2 or 3 modulates receptor function in non-transfected cells.

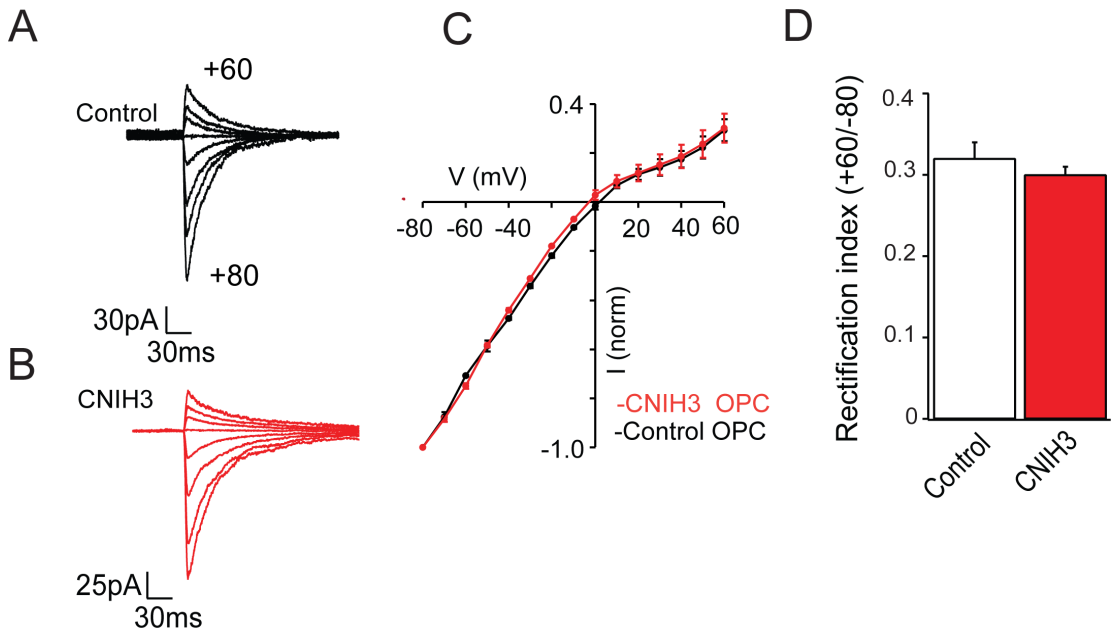


Figure 5.4. CNIH3 overexpression does not alter AMPAR rectification in OPCs.

(A, B) AMPAR currents induced by application of 10mM glutamate to outside-out patches from control and CNIH3 transfected**. (C)** Inwardly rectifying *I-V* plots from AMPARs in responses to fast application of glutamate (10mM, 100ms) to outside-out patches excised from OPCs. Currents were examined between -80 and +60mV (n=3 control; n = 3 CNIH3 overexpressed) (100μM spermine present in the intracellular solution)**. (D)** Overexpression of CNIH3 did not modify the rectification of responses (control RI= 0.34 ± 0.02, and 0.3 ± 0.06 following CNIH transfection; n = 3, n = 3, P = 0.29 unpaired t-test), indicating the presence of GluA2 containing and GluA2 lacking AMPARs in both conditions.

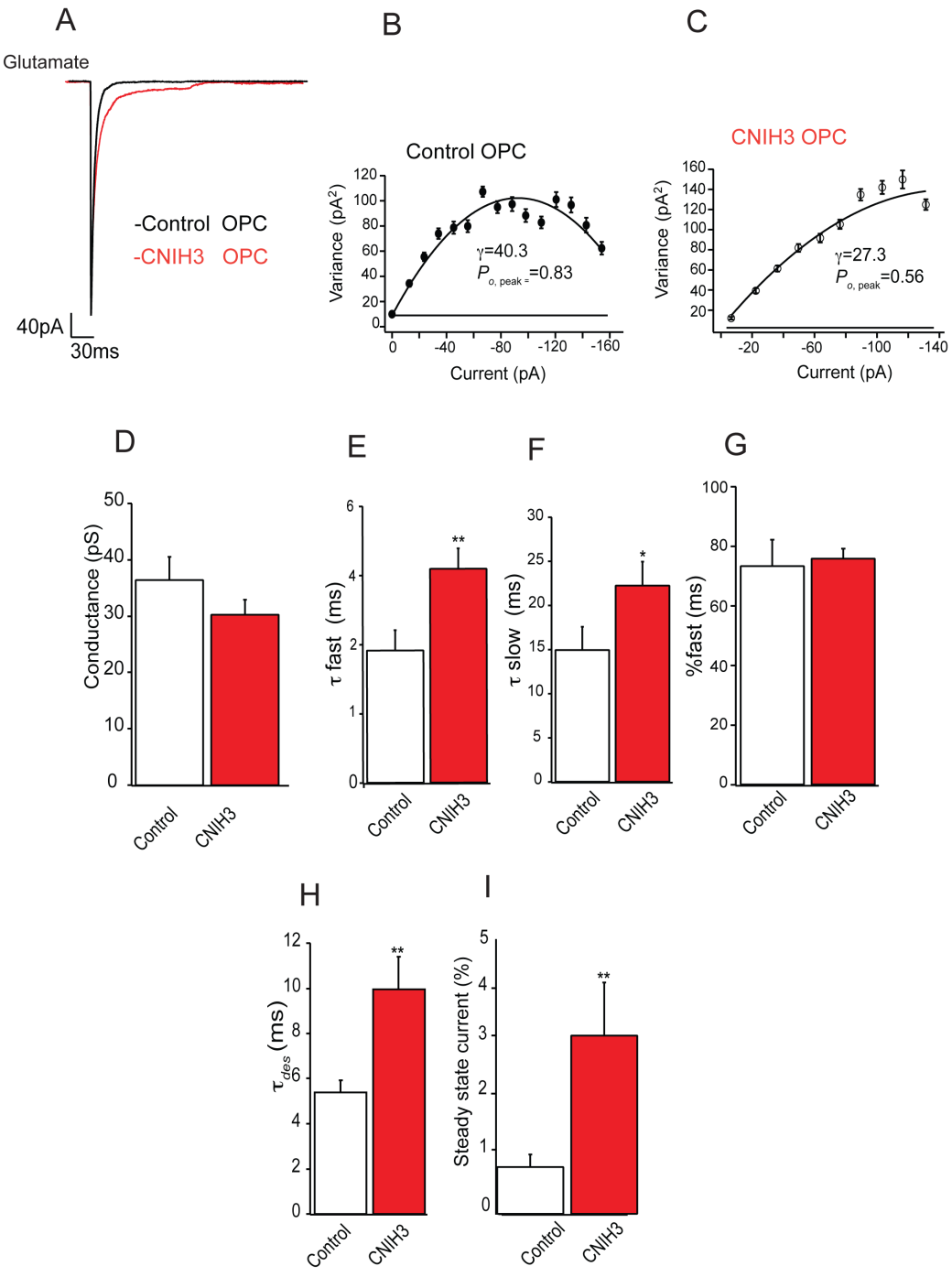


Figure 5.5. CNIH3 decreases AMPA channel desensitization in optic nerve OPC glia.

(**A**) Glutamate evoked responses recorded from optic nerve OPC DIV 7 from control (n = 11) and from OPCs transfected with CNIH3 (n = 10). Mean of 45 traces from control and mean 94 from CNIH3 transfected cells. Traces obtained from outside-out patches by rapid application of 10mM glutamate (100ms, +60mV). **(B-D)** Current variance plots from control and CNIH3 transfected OPCs. The estimated single-channel conductance was not significantly modified by

CNIH3 (control cells 35.3 ± 3.7pS CNIH3 transfected 29.06 ± 2.3pS (P = 0.09). **(E-I)** Overexpression of CNIH3 significantly decreased AMPAR channel desensitization from control τ_W 5.31 ± 0.54ms to 9.87 ± 1.39ms in CNIH3 transfected cells (P = 0.007**) and increased the AMPAR percentage steady state current from control 0.56 ± 0.02 to 2.98 ± 0.07 for CNIH3 transfected cells (P = 0.031**).

5.3.4. CNIH3 increases kainate induced AMPAR responses from OPCs

In order to further evaluate the effects of CNIH3 on OPC AMPARs, we used a previously established assay to investigate the effects of TARPs on AMPAR pharmacology. The assay measures the different currents produced by the partial agonist kainate compared with glutamate (I_{KA}/I_{Glu}). This method has been used by Shi et al*.,* 2009 to show that when TARPs are co-expressed with AMPARs the relative size of the AMPAR response is increased when activated with 1mM kainate, compared to 1mM glutamate (I_{KA}/I_{Glu}). It would therefore be interesting to understand if CNIH3 acts as a TARP and modifies the kainate induced AMPARs current when CNIH3 is overexpressed in OPCs.

Initially we examined the I_{KA}/I_{Glu} ratio for untransfected OPC AMPARs by measuring activation with 1mM kainate and 1mM glutamate from outside-out patches in the presence of 100μM cyclothiazide (to prevent AMPAR desentisation). Previous studies have shown that in expression systems the I_{KA}/I_{Glu} ratio for GluA1 alone was 0.02 and significantly increased to 0.49 when co-expressed with γ-2 (Shi et al., 2009). Furthermore, a very recent study has shown that the I_{KA}/I_{Glu} ratio for GluA1 when co-expressed with CNIH2 is on average increased from 0.02 in control to 0.1 with CNIH2 (Shi et al., 2010). We found that the I_{KA}/I_{Glu} ratio for control OPCs were similar to the published I_{KA}/I_{Glu} ratio for GluA1+γ2 suggesting that the

majority of cell surface AMPAR present on OPCs are already associated with γ-2 (Control OPC I_{KA}/I_{Glu} ratio = 0.415 ± 0.04 n = 7) (**Figure 5.6A**). However to examine whether we could provide further evidence that an overexpression of CNIH3 can modify the properties of cell surface AMPARs on OPCs, we measured the I_{KA}/I_{Glu} ratio for OPCs transfected with CNIH3, and found that in CNIH3 transfected OPCs cells the AMPAR I_{KA}/I_{Glu} ratio significantly increased to 0.786 ± 0.1 n = 4 compared to control values of 0.414 ± 0.04) (P = 0.023) (**Figure 5.6B**). This increase in I_{KA}/I_{Glu} ratio for CNIH3 transfected OPCs was unexpected, as previous studies in expression systems have shown that GluA1+CNIH3 I_{KA}/I_{Glu} ratio to be 0.1. The fact that I_{KA}/I_{Glu} ratio is higher than would be expected might indicate co-operation between cornichons and TARPs, suggesting that the two auxiliary proteins can bind to the same receptor. To investigate this we have carried out preliminary studies in TSA201 cells transfected with GluA1+γ-2+CNIH3, and found that the I_{KA}/I_{Glu} ratio may be increased to 0.592 (n = 2) compared to GluA1+γ-2 alone. This suggests that when AMPARs are associated with a mixture of TARPs and cornichons, there is an increase in the AMPAR response to kainate.

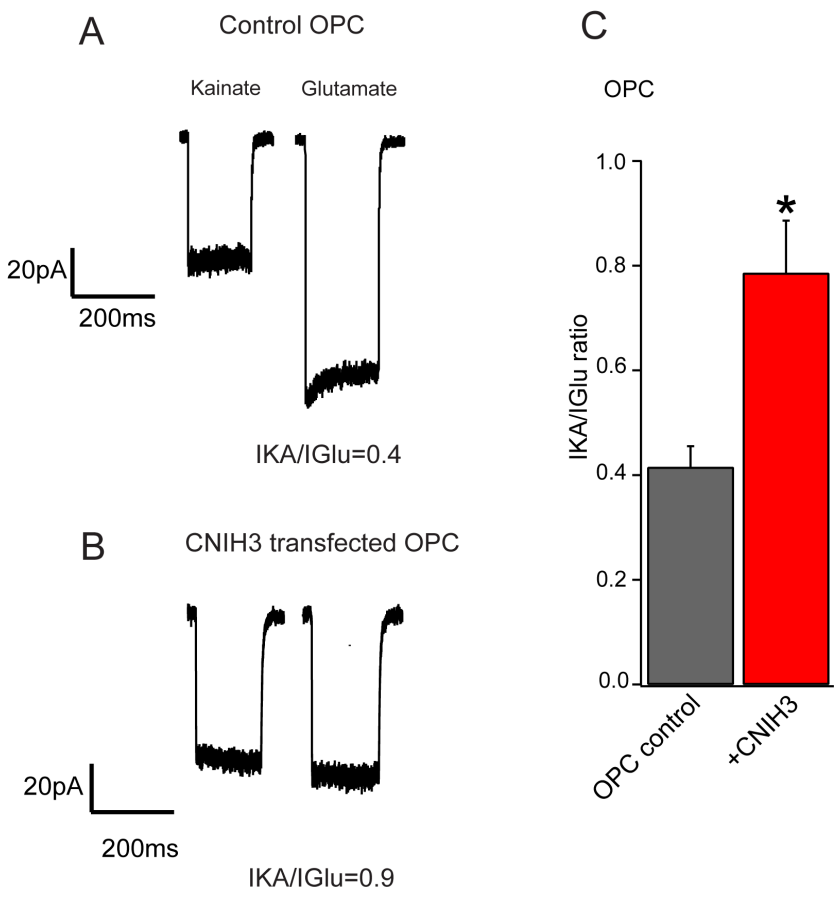


Figure 5.6. CNIH3 overexpression enhances kainate induced AMPAR currents in OPC.

(A-B) Typical example of Kainate- (KA, 1 mM) and glutamate- (Glu, 1 mM) induced currents produced using outside-out patches from control OPC and OPC transfected with CNIH3. (**C)** CNIH3 significantly enhanced the I_{KA}/I_{Glu} ratio compared to control I_{KA}/I_{Glu}= 0.415 ± 0.04 n = 7 to I_{KA}/I_{Glu} CNIH3 = 0.786 ± 0.1 n=4 (P = 0.023*).

5.4. Discussion

The aims of this study were to determine whether CNIH2/3 could modify the properties of AMPARs in glia. We have shown that CNIH2/3 are expressed in cell surface of glial cells from the cerebellum and optic nerve. Furthermore by overexpressing CNIH3 in OPCs we find that in contrast with the situation in neuron, CNIH3 can modulate the properties of cell surface AMPARs. This study therefore provides evidence that CNIH3 may

be capable of controlling the functional properties of cell surface AMPARs in a cell-type specific manner.

Previously we have shown that AMPARs in glia are regulated by TARPs (Soto et al., 2009). In particular γ-5 regulates communication between neurons and Bergmann glia (**Chapter 3 Figure 4.4**). From the experiments described in the present chapter, it appears that cornichons are also expressed on the cell surface of glia and are capable of modifying AMPAR properties when overexpressed. An important question is whether AMPARs bind to both cornichons and TARPs in glial cells. To address this, further work will be needed, as our understanding of the stoichiometry of AMPARs, CNIHs and TARPs in glial cells remains unknown.

The dramatic change in the kinetic properties of AMPARs when CNIH3 is overexpressed in OPCs suggests that either cornichons compete with other auxiliary proteins and displace them or alternatively that CNIH3 form a complex with other auxiliary proteins and AMPARs. Interestingly overexpression of CNIH3 did not modify the rectification properties of AMPARs suggesting that it does not selectively modify the trafficking of CP-AMPARs. Consistent with this, we did not observe a change in the single-channel conductance in CNIH3 transfected cells.

It has been suggested in a recent study that CNIH2 is not present at the cell surface of granule cells and is localized mainly in the Golgi (Shi et al., 2010). This raises the question as to why overexpression of cornichons can modify the cell surface properties of AMPARs in glial cells and not in neurons. The lack of change in the properties in CNIH2 transfected neurons could be due to the following: (i) there could be a more stringent secretory pathway in neurons, which could be lacking glia. This would allow the trafficking of 'cornichoned' AMPARs to the cell surface of glia and in

expression systems, (ii) CNIH3 could only modify the properties of specific AMPAR subunits *in vivo*. OPCs express a mixture of GluA2, 3 and 4 whereas both cerebellar granule cells and hippocampal neurons express short form GluA2/4 heteromers, (iii) neurons express a wide variety of synaptic proteins, including PSD-95, which are important for regulating synaptic plasticity and have roles in stabilizing AMPARs at the cell surface. However it is possible that CNIH3 can traffic to the cell surface of OPCs due to the expression of a unique trafficking protein that may be absent in neurons resulting in AMPAR bound to CNIH2 or CHIN3 remaining in the TGN.

Shi et al., 2009 demonstrated that the expression of a covalently linked TARP-AMPAR complex significantly increase AMPAR responses to kainate. When overexpressing the TARP γ-8 in hippocampal neurons the I_{KA}/I_{Glu} was significantly increased. In this study I have shown that an increase in CNIH3 expression in OPCs also produces an increase in the AMPAR response to kainate, thus suggesting that the cell surface receptors are coupled to CNIH3, which has the ability to modify the functional properties.

6. General discussion

The experiments described in this thesis I have identified novel proteins and intracellular pathways potentially important for controlling glial AMPARs.

In Chapter 3 we identified that the novel TARP γ-5 regulates the properties of AMPARs in BGC and is associated with the protein SAP-97. Interestingly, in contrast with other established TARPs, γ-5 suppresses the cell surface trafficking of AMPARs, which is regulated through the lysosomal pathway. We also found that, unlike other TARPs, γ-5 appears to be highly localised to glial cells, being present in cerebellar BGC and in glia of the olfactory bulb and spinal cord. It would clearly now be of interest to determine whether γ-5 is involved in regulation of AMPARs in spinal cord and olfactory bulb glial cells. There is evidence for the presence of ensheathing glial cells (OEC) in the olfactory bulb. Like Bergmann glia in the cerebellum OECs also seem to be important for axon guidance. Indeed, OECs have been linked to increased neuronal cell survival (Au et al., 2007). It would be of interest to investigate whether OECs express CP-AMPARs, and whether γ-5 regulates the interaction between OECs and olfactory neurons. Methods similar to those used by Iino et al. (2003) in their experiments on BGCs could provide useful information on the importance of CP-AMPARs in OECs.

While CP-AMPARs and TARPs play important roles in healthy glial cells, their role in glioma proliferation and growth is less clear (Jansen et al., 2010). Gliomas have been found to express various AMPAR subunits (GluA1, GluA3 and GluA4), which give rise predominantly to CP-AMPARs. When activated these can lead to enhanced proliferation of glial tumours

through the Ras/Raf/MEK-1 pathway (Beretta et al., 2009). Several studies have shown that a downregulation of GluA1-containing CP-AMPARs reduces growth and proliferation of gliomas (de Groot et al., 2008). Indeed, the proposed AMPAR antagonist talampanel is currently in phase 2 clinical trials for treatment of gliomas (Iwamoto et al., 2010).

It has also been shown that there is increased expression of the unedited GluA2(Q) in glial tumour cells and that activation of unedited AMPARs influences cells proliferation (Ishiuchi et al., 2007). There is currently no published work on the role of TARPs in glioma growth. It seems quite likely that properties of CP-AMPAR in gliomas are controlled by γ-5 and that knockdown of this TARP (for example with sRNAi) could potentially reduce the cell surface expression of CP-AMPARs to reduce the rate of proliferation and migration of gliomas.

In chapter 4 we described experiments on optic nerve OPCs and found that, like neurons, these can undergo mGluR dependent AMPAR plasticity, which is regulated by the mTOR pathway and blocked by cyclohexamide (C-hex). Furthermore, ATP modulated the plasticity of AMPARs in OPCs and decreased their single-channel conductance, Surprisingly this effect on AMPARs was not inhibited by C-hex..

The experiments described in Chapter 5 identify cornichon proteins in the surface of glial cells in the cerebellum and optic nerve. In light of recent work (Shi et al., 2010) it was surprising that we found CNIH3 was able to modify the properties of AMPARs when overexpressed in OPCs. Examination of the possible role of cornichons in glial cells would be facilitated if knockout mice were available in which CNIH2/3 knockdown

was under the control of a glial cell promoter. This might allow the identification of potential abnormalities in glial cell AMPAR properties.

Recently abnormalities in the expression in the genes encoding the TARPs γ-2 have been linked with epilepsy and schizophrenia (Kato et al., 2008). However, it is currently unknown whether there is a correlation between mutations in genes encoding cornichons and specific neurological disorders. Interestingly, a study using gene microarray studies of animal models of schizophrenia has identified a reduced expression levels of CNIH2 in the cerebellum (Fatemi et al., 2008), raising the possibility that cornichons may be important in neuronal-glial communication as described for TARPs in this thesis.

The importance of glia in facilitating LTP in neurons, and the role of proteins which regulate neuronal-glial communication, have become the focus of many recent studies (Haydon et al., 2009; Henneberger et al., 2010; Zhang et al., 2009). With the advent of microarray studies we can now detect changes in the expression levels of many proteins linked to AMPAR trafficking. It is anticipated that this will provide further vital information on how glial cells, and their AMPARs and TARPs, are regulated throughout the CNS. Clearly, further studies are required to provide greater insight into how AMPARs are trafficked and how channel properties modified in glia.

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